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REVIEW

Gender specific medicine in liver diseases: A point of view

Marilena Durazzo, Paola Belci, Alessandro Collo, Vanessa Prandi, Erika Pistone, Maria Martorana, Roberto Gambino, Simona Bo

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Abstract

Gender medicine focuses on the patho-physiological, clinical, prevention and treatment differences in diseases that are equally represented in men and women. The purpose of gender medicine is to ensure that each individual man and woman receives the best treatment possible based on scientific evidence. The concept of "gender" includes not only the sexual characteristics of individuals but also physiological and psychological attributes of men and women, including risk factors, protective/aggravating effects of sexual hormones and variances linked to genetics and corporal structures that explain biological and physiological differences between men and women. It is very important to consider all the biological, physiological, functional, psychological, social and cultural characteristics to provide patients with individualized disease management. Herein, we critically analyze the literature regarding gender differences for diseases and acquired conditions of the most representative hepatic pathologies: primary biliary cirrhosis, autoimmune hepatitis, primary sclerosing cholangitis, non alcoholic fatty liver disease and alcoholic liver disease, and viral chronic hepatitis B and C. The last section addresses hemochromatosis, which is a prevalent iron overload disorder in the Caucasian population. This review aims to describe data from the literature concerning viral chronic hepatitis during pregnancy, management during pregnancy and delivery, and new effective drugs for the prevention of maternal infection transmission without significant adverse effects or complications.

Key words: Gender; Liver disease; Primary biliary cirrhosis; Autoimmune hepatitis; Viral chronic hepatitis B; Viral chronic hepatitis C; Non alcoholic fatty liver disease; Alcoholic liver disease

Core tip: Gender medicine focuses on the patho-physiological, clinical, prevention and treatment differences in diseases that are equally represented in men and women. The concept of "gender" includes not only the sexual characteristics of individuals but also physiological and psychological attributes of men and women. In this review, we critically analyze the literature regarding gender differences for diseases and acquired conditions of the most representative hepatic pathologies: primary biliary cirrhosis, autoimmune hepatitis, primary sclerosing cholangitis, non alcoholic fatty liver disease and alcoholic liver disease, viral chronic hepatitis B and C, and hemochromatosis (the prevalent iron overload disorder in the Caucasian population).

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INTRODUCTION

Gender medicine is a new aspect of medicine that focuses on to investigating the differences in diseases based on anatomic and physiological stages, from biological, functional, psychological, social and cultural points of view and analyzes the range of responses to pharmacological care. This field emerged because epidemiological and clinical surveys performed over the last 30 years have generally reported results for only gender[1].

The concept of "gender" refers not only to the sexual characteristics of individuals, but also to a set of differences derived from the physiology and psychology of men and women and from various social and cultural environments. From biological and physiological points of view, the differences between men and women may be explained by differences in the presence of risk factors, protective/aggravating effects of sexual hormones, variances linked to genetics and various corporal structures[2].

The aim of this review is to examine the available data in the literature concerning the differences between men and women for the most representative hepatic pathologies, including primary biliary cirrhosis (PBC), autoimmune hepatitis (AIH), viral chronic hepatitis B and C, non alcoholic fatty liver disease (NAFLD) and alcoholic liver disease. There are morphological differences in the liver between genders. Thus, hepatic damage may produce different consequences in men and women in ongoing primitive diseases and during acquired conditions[2].

AUTOIMMUNE LIVER DISEASES: PRIMARY BILIARY CIRRHOSIS, AUTOIMMUNE HEPATITIS AND PRIMARY SCLEROSING CHOLANGITIS

Previous studies have examined gender differences in the immune system, and suggest that estrogen and androgen may modulate the immune system. Women have a significantly higher number of $CD4^+$ T lymphocytes and a higher $CD4^+/CD8^+$ ratio than men[3].

Additionally, the secretion of interferon-γ (IFN-γ) and interleukin (IL)-10 was enhanced after the addition of estrogen in T-cell clones isolated from women[4]. Conversely, androgen inhibited, the secretions of IFN-γ, IL-4, and IL-5 in murine T cells[5].

These findings suggest that gender differences could have a role in autoimmune diseases.

The best example demonstrating gender differences is PBC.

PBC is a chronic cholestatic liver disease characterized by immune-mediated inflammatory destruction of the small intrahepatic bile ducts, and fibrosis. PBC can progress to cirrhosis and subsequent liver failure[6-8]. PBC is a typical female disease that occurs from 40-60 years of age[9]. The incidence rates in women and men range from 3:1 to 22:1, with an average incidence rate in women of 10:1[10]. The age at PBC diagnosis was found to be older in men (62 years) than in women (51 years)[11].

Numerous hypotheses have been formulated to justify this sex imbalance. For example, the effects of sex hormones in lymphocyte maturation/activation and the synthesis of antibodies and cytokines have been suggested as contributing factors. Additionally, the immunemodulatory effects of estrogens during reproductive life, fetal microchimerism, skewing of the X-chromosome inactivation pattern and defects in sex chromosomes have also been suggested as factors[12]. Several studies have identified an increased incidence of X haploinsufficiency in female patients[13,14].

A study by Selmi *et al*[15] indicates that epigenetic factors, such as X chromosome inactivation, may also be involved in the development of PBC, and variable concordance rates of PBC have been identified between twins. A recent study by Lleo *et al*[16] demonstrated how Y chromosome loss is associated with PBC in male patients. These epigenetic changes may be ideal targets for new personalized treatments, as suggested by cancer data. However, no convincing evidence has yet supported any of these hypotheses. Males are less likely to be symptomatic than females. Females experience pruritus as a single symptom more often than males. It has been suggested that female sex hormones may be linked with pruritus. In addition, female sex hormones may cause more abdominal pain/discomfort and constitutional symptoms (malaise, anorexia, weight loss, fatigue). In contrast, males experience more jaundice, jaundice with pruritus, and upper gastrointestinal bleeding[17].

The rates of severe daytime somnolence and depressive symptoms were found to be similar in males and females; in contrast, autonomic symptoms were more profound in females[18,19].

Concomitant autoimmune diseases such as, Sicca Syndrome, Scleroderma and Raynaud's phenomenon, were shown to be less prevalent in men. These findings suggest that females are more likely to suffer concomitant autoimmune disease than males. The complications of hepatocellular carcinoma (HCC) in patients with PBC were reported to be significantly greater in men than in women[20]. Biochemical levels of alkaline phosphatase (ALP), alanine aminotransferase (ALT) and gamma-glutamyl transpeptidase (gGT) were reported to be slightly higher in symptomatic males compared to asymptomatic males, but both were higher than in females[21]. The only histological difference identified were that symptomatic female patients had more piecemeal necrosis of the liver and that symptomatic males had more stainable copper storage than asymptomatic males. Additionally, symptomatic females were reported to have more pseudoxanthomatous transformation than asymptomatic females[17]. AIH is a liver disease characterized by progressive inflammatory destruction of the parenchyma. AIH is associated with the presence of circulating autoantibodies, hypergammaglobulinemia and interface hepatitis on liver biopsy. AIH typically responds to immunosuppressive therapy[22].

Table 1 Gender differences in primary biliary cirrhosis and autoimmune hepatitis

Primary biliary cirrhosis	Autoimmune hepatitis
M/F ratio 1:10	M/F ratio 1:3.6
Age at diagnosis higher in M than in F (62 yr *vs* 51 yr)	Normalization of ALT levels after 6 mo of corticosteroid treatment less frequent in M than in F
M less symptomatic than F: pruritus, abdominal pain/ discomfort and constitutional symptoms more common in F; jaundice and upper gastrointestinal bleeding more common in M	Better long-term survival and outcome in M than F
Concomitant autoimmune diseases more common in F (sicca syndrome, sclerodermia, raynaud phenomenon), whereas HCC complication are significantly greater in M	Decrease of severity during second trimester of pregnancy and possible onset of acute exacerbation after delivery
ALP, ALT and gGT higher in M than F	Haplotype HLA A1-B8-DR3 more prevalent in M than in F
Piecemealnecrosis and pseudoxanthomatous trasformation greater in symptomatic F	Higher frequency of concurrent immunological disorders at presentation in F than M

HCC: Hepatocellular carcinoma; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; gGT: Gamma-glutamyl transpeptidase; HLA: Human leukocyte antigen; F: Female; M: Male.

The etiology of AIH is unknown, though both genetic and environmental factors are involved. It has been suggested that the major mechanism of liver damage is the failure of impaired regulatory T cells to control immune reactions against liver host antigens.

The actual prevalence of AIH is unknown. AIH is characterized by a strong female preponderance (the female/male ratio is 3.6/1)[23]. There are no sex or gender differences in age, form of clinical onset, frequency of symptomatic concurrent autoimmune diseases, and human leukocyte antigen DR (HLA DR) status. Several studies have demonstrated that in men, there is a minor frequency of normalization of ALT stages after 6 mo of corticosteroid treatment[24]. However, men with AIH appeared to have better long-term survival and outcome than women[25]. In females the severity of AIH was found to be likely to decrease during the second trimester of pregnancy, when estrogen was secreted at high levels and acute AIH exacerbation occurred occur after delivery[26]. High levels of estrogen are associated with an anti inflammatory milieu[27]. Moreover females have a higher frequency of concurrent immunological disorders such as Sicca Syndrome at presentation than males.

Al-Chalabi *et al*[25] discovered the extended haplotype HLA A1-B8-DR3 (associated with increased susceptibility to AIH) was more than twice as prevalent in male patients as in female patients with AIH.

Primary sclerosing cholangitis (PSC) is a chronic cholestatic liver disease characterized by progressive inflammation and fibrosis of the intrahepatic andextrahepatic bile ducts. PSC leads to cholestasis, progressive hepatic fibrosis and eventually decompensated cirrhosis[28,29].

The incidence of PSC is 1:100000 people. Previous studies have demonstrated that PSC is more prevalent in men than in women (M > F 7:3). In the United States, between 62% and 70% of patients are male[30]. The pathogenesis of PSC is unclear because it is a complex immune-mediated disease. The most accepted theory is that in genetically predisposed individuals, the environmental exposure to infective agents or toxins causes persistent immunemediated damage to cholangiocytes and progressive destruction of bile ducts, which leads to chronic cholestasis[31] (Table 1).

ALCOHOLIC LIVER DISEASE

Alcohol abuse and its various complications are still widespread in the Western world and represent a frequent cause of hepatic damage. The excessive consumption of alcohol may cause hepatic steatosis, alcoholic hepatitis and cirrhosis. Alcoholic cirrhosis causes approximately 40% of deaths due to cirrhosis. The severe forms (hepatitis, cirrhosis) are associated with ingestion of 160 g/die of alcohol in 10-20 years. The incidence of alcoholic liver disease increases proportionally with the consumption of alcohol. Several surveys have demonstrated that hepatic damage develops faster in women than in men. In cases of heavy drinkers with a weekly consumption of 336-492 g, the relative risk of developing cirrhosis was equivalent to 7 in men and 17 in women. Furthermore, the relative risk of developing alcoholic liver disease was 3.7 in men and 7.3 in women. The factors regulating in the differences in susceptibility to alcoholic toxicity include the following: age during alcohol consumption, the manner of alcohol consumption (with or without meals) and the nutritional state of the individual[32].

Women are more susceptible to damage by alcohol compared to men, which leads to more advanced liver disease after alcohol consumption. It has been demonstrated that, under the same conditions and assuming equal doses of alcohol, women reach higher blood ethanol concentrations than men. Moreover, it has been shown that females have a major risk of hepatitis progression toward cirrhosis after abstaining from alcohol[33,34]. The causes attributed to these gender differences include differences in corporal structures, different enzymatic activity and hormonal differences.

The process of metabolizing a substance before it enter the general circulation is called first-pass metabolism. Various studies have demonstrated that an isoform of gastric alcohol dehydrogenase (ADH) has a main role in alcohol metabolism. ADH activity is linked to the first passage of alcoholic metabolism and affects the blood ethanol concentration. At the gastric level, this enzyme is expressed less in women than in men. Furthermore, in a female heavy drinker the activity of gastric ADH is practically absent. Therefore in women a majority of alcohol reaches the liver directly, which may worsen the hepatic damages. Moreover, this situation contributes to the gen-

Table 2 Gender differences in alcoholic liver disease

Alcoholic liver disease (hepatic steatosis, alcoholic hepatitis, cirrhosis)
Hepatic damage faster in F than M
RR to develop cirrhosis 7 in M and 17 in F
RR to develop alcoholic liver disease 3, 7 in M and 7, 3 in F
F more susceptible to damage by alcohol than M: higher haematic concentration of ethanol in F than M: major risk of hepatitis progression toward cirrohosis (even after an absentation from alcohol) in F than M
Differences in corporal structures (content of corporal water), different enzymatic activity (gastric ADH expression and activity), hormonal

ADH: Alcohol dehydrogenase; RR: Relative risk; F: Female; M: Male.

der differences in blood concentration and contributes to unfavorable consequences of alcohol use[35]. Another cause of female vulnerability to the toxic effects of alcohol is the reduced content of corporal water compared to men[36].

The quantity of absorbed alcohol in the gastro-intestinal system that is not metabolized by first-pass metabolism enters the circulation. Hepatic ADH, in the liver is principally involved in alcohol metabolism. The amount of alcohol distributed in water determines the blood alcohol concentration. A woman has proportionally more fat and less water than a man. Thus, when the ethanol is distributed in water, the distribution volume in women is less, and the blood alcohol concentration is higher[35,37] (Table 2).

NAFLD

NAFLD is the most common chronic liver disease in the Western world, affecting 30% of the general adult population[38].

NAFLD is an umbrella term for a group of diseases defined by a hepatic fat infiltration in > 5% of hepatocytes, in the absence of excessive alcohol intake. Excessive alcohol intake is defined as two standard drinks (20 g ethanol) daily for men and one standard drink (10 g ethanol) daily for women. NAFLD encompasses a histological spectrum ranging from simple steatosis to non-alcoholic steatohepatitis (NASH). NASH is defined by steatosis, hepatocellular damage and lobular inflammation[39] in individuals without significant alcohol consumption and without viral, congenital and autoimmune liver disease markers.

There have been parallel increases in the prevalence rates of obesity, metabolic syndrome (hyperglycemia, visceral obesity, hyperlipidemia and hypertension) and NASH. As a result, NASH is considered part of metabolic syndrome (MS)[40].

MS is a risk factor for cardiovascular disease, its high prevalence has substantially affected public health in recent years[41]. There are varied reports in the literature regarding the gender distribution of MS. Several studies report a higher incidence of MS in men than in women, but the reverse has been shown in other reports[42].

The prevalence of MS increases with the general population age and is more likely in black and Hispanic female populations. The accumulation of hepatic and intra-

Table 3 Non alcoholic fatty liver disease and gender

NAFLD and gender
Prevalence of MS in men and postmenopausal women
Prevalence of visceral adiposity in men and postmenopausal woman
Possible link to MS, NAFLD and sex hormones

NAFLD: Non alcoholic fatty liver disease; MS: Metabolic syndrome.

abdominal fat is not different between genders, but it is affected by dietary lipid consumption[43]. Abdominal fat tissue is a major source of free fatty acids and cytokines for the liver, and fat favors the early development of insulin resistance, dyslipidemia, and high blood pressure. The more favorable fat distribution in women demonstrates why women need a higher degree of adiposity to achieve the same metabolic disturbances as men[44]. Subcutaneous and visceral adipose tissue types are influenced by age and gender. Visceral adipose tissue accumulates more rapidly with age and weight gain in males and postmenopausal females than in younger females[45]. The difference in the prevalence of MS between genders has been attributed to sex hormones. Many studies have shown that, in postmenopausal women, the distribution of their body fat changes toward visceral adiposity[46]. There are differing reports in the literature concerning the association between gender and NASH. In some studies, NAFLD was approximately 1.5 times more prevalent in females than in males whereas other studies did not find any differences, between genders[47,48]. Some studies have shown that female gender is a risk factor for NASH[49], but the current literature presents conflicting results[50,51]. A population, based study suggested that endogenous estrogens have a protective role in NASH, which may explain why the prevalence of NAFLD increases in women over 50 years of age[52].

In conclusion, although the sex differences for fibrosis in patients with NAFLD are not identical, women tend develop more, severe fibrosis than men[53].

The possible roles of estrogen in hepatic lipid metabolism and fibrosis require further investigation (Table 3).

CHRONIC HEPATITIS B

Protracted treatment with nucleoside/nucleotide analogs has allowed for an improvement in the natural history of patients with chronic hepatitis B virus (HBV) infection by reducing the incidence of cirrhosis and the risk of complications[54].

Over the last 20 years, the epidemiology of HBV infection has radically changed in Italy. At the beginning of the 1980, the rate of HBV surface antigen (HBsAg) carriers in the general population was 3.5%, with peaks of 10% in Southern Italy. The current prevalence of carriers is less than 1%, and a majority of carriers are male. The rate of chronic infection is higher in men due to various factors and is widely studied. However, it is unclear if men are exposed to more viruses, or if men have a less effective immune response in eliminating. The major re-

Table 4 Chronic hepatitis B during the pregnancy and in the foetus

HBV and pregnancy	HBV and foetus
Not increases in maternal morbidity and mortality	Maternal transmission: during delivery, intrauterine transmission and during breast feeding
Increases HBV viremia levels and indices of cytolysis	Discordant results from pre-delivery administration of Ig and anti-HBV vaccine
Development of complications (gestational diabetes, pre-delivery hemorrhages and pre-term delivery)	Administration of Ig and anti-HBV vaccine during delivery to prevent infection
Higher frequency of gestational hypertension, detachment of placenta and peripartum hemorrhages in F with cirrhosis	Ongoing studies about the use of antiviral medicines in F with high HBV DNA levels to prevent perinataltransmission (telbivudine and tenofovir in FDA pregnancy category B)
Cases of peripartum hepatitis with hepatic decompensation	

HBV: Hepatitis B virus; F: Female; FDA: Food and drug administration.

sponse in females is caused by the position of genes that determine the response, and most genes are located on the X chromosome. This hypothesis is supported by the female prevalence of two hepatic autoimmune diseases (PBC and AIH)[55].

HBV does not meaningfully influence fertility, and contracting an HBV infection during a pregnancy does not increase morbidity or maternal or fetal mortality[56]. Recent evidences demonstrated that the increased production of proinflammatory cytokines in chronic hepatitis B (CHB)[57,58] may participate in the development of complications, such as gestational diabetes, pre-delivery hemorrhages and pre-term delivery[59]. Furthermore, in women with cirrhosis, there are higher frequencies of gestational hypertension, detachment of the placenta and peripartum hemorrhages compared with healthy controls[60].

A normal pregnancy with elevated levels of corticosteroid hormones and estrogens cause increased HBV viremia[61] and indices of cytolysis (ALT)[62]. Moreover, there have been reported cases of peripartum hepatitis with hepatic decompensation[63].

The main cause of fetal HBV transmission is delivery. The administration of immunoglobulins and an anti-HBV vaccine may prevent fetal infection in more than 85% of children born from $HbsAg^+$ mothers[64].

Other minor causes of fetal and maternal transmission are intrauterine transmission (HBV may reach the foetus through the placental barrier)[65] and transmission during breastfeeding through virus ingestion or by contact with maternal cutaneous lesions[66].

There is currently no clear therapeutic way to prevent viral transmission. The pre-delivery administration of immunoglobulins has yelded discordant results[67,68]. The study by Beasley demonstrated that the administration of immunoglobulins and anti-HBV vaccine within 12 h of birth reduced the frequency of HBV transmission from > 90% to 26%[69,70].

A 2012 Chinese study evaluated the safety of lamivudine treatment for CHB in early pregnancy. This study examined 92 chronic HBV-infected pregnant women who received Lamivudine treatment either before pregnancy or in early pregnancy. These women were not co-infected with hepatitis C virus (HCV), human immunodeficiency virus, cytomegalovirus, or other viruses. Adverse events were observed throughout the entire pregnancy and perinatal period. The effectiveness of Lamivudine treatment for blocking mother-to-infant transmission of HBV was evaluated. The data showed that treatment does not increase complications or adverse events for mothers during pregnancy or the perinatal period. Additionaly no effect on fertilization or embryonic development was found, and treatment did not increase the incidence of congenital abnormalities in infants. Furthermore, treatment reduced the rate of mother-to-infant transmission[71]. A case report described a treatment with triple therapy of Lamivudine, IFN-beta and prednisolone for acute CHB exacerbation during pregnancy. The patient's liver enzymes became elevated toward the end of the first trimester. She was treated with Lamivudine, interferon-beta and steroids early in the second trimester. After this treatment, aminotransferase levels rapidly normalized within 4 wk. Lamivudine was continued until delivery. Spontaneous delivery occurred at 37 wk of gestation. There were not congenital anomalies, and fetal growth was found to be within normal reference ranges. This case report suggests that combination therapy with Lamivudine, IFN-beta and steroids may be safely used during the pregnancy to treat acute CHB exacerbations[72].

There are ongoing studies investigating the use of antiviral medicines in mothers with high HBV DNA levels. Currently the oral antivirals Telbivudine and Tenofovir are classified as "FDA pregnancy category B", whereas the other antiviral drugs are classified as "FDA pregnancy category C". A recent meta-analysis has demonstrated that Telbivudine use in the final stage of pregnancy is effective in preventing or reducing the perinatal transmission of HBV without meaningful or unfavorable effects[73].

Some data exist on tenofovir in HIV positive women but these data show increased congenital malformations, kidney damage and distorted bone metabolism after exposure *in utero*[74] (Table 4).

CHRONIC HEPATITIS C

HCV infection affects 130-170 million people worldwide, which is approximately 2%-3% of the global population. HCV is transmitted by parenteral routes, such as contact with infected blood or contaminated materials and intravenous drugs injection with contaminated syringes. Although less common, HCV can be transmitted by sexual contact with HCV-positive partners[75,76]. Several studies have demonstrated that women have less altered hepatic biochemical tests and lower rates of fibrosis progression[77]. These findings are related to the protective effects of estrogens, which possess anti-fibrotic properties. Estrogens have a role in blocking fibrogenesis in hepatic

Table 5 Chronic hepatitis C during the pregnancy

Chronic hepatis C and pregnancy
Frequency of HCV MTCT is 5%-10%
Vertical transmission is the main cause of pediatric HCV infection
Factors increasing the risk of MTCT: amniocentesis, extended breaking of the membranes and elevated viral load in the mother
High levels of ALT in the previous year of pregnancy are linked with a higher MTCT rate
Signs of viral replications is maternal peripheral blood mononuclear cells enhance vertical transmission
Breastfeeding and genotype are not linked to MTCT
Presence of HCV-HIV coinfection increases MTCT by 90%
The administration of combined therapy is not recommended during pregnancy

HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; MTCT: Mother-to-child transmission; ALT: Alanine aminotransferase.

stellate cells. The notion that estrogen has a protective role was also suggested by evidence that menopause is associated with an accelerated rate of fibrotic progression and that hormone replacement therapy may minimize this effect[78]. The prevalence of HCV infection in pregnancy is 1%-2% in the United States and Europe. However, the rate of HCV, may reach up to 8% in some developing countries[79].

The documented mother-to-child transmission (MTCT) frequency of HCV is approximately 5%-10%[80]. The pathogenesis of HCV infection during pregnancy and the neonatal period is unclear. During pregnancy, the maternal immune system has to develop tolerance to paternal antigenes to avoid any maternal immune assault towards the fetus. Simultaneously, the maternal immune system most maintain active immunity against HCV to protect both the mother and fetus from infection. This modulation of immune responses is different during each stage of pregnancy[81]. In developed countries, vertical transmission is the main cause of pediatric-HCV infection. The factors demonstrated to increase the risk of maternal-fetal transmission include amniocentesis, the extended breaking of the membranes and an elevated viral load in the mother. Perinatal HCV transmission is confined to women with HCV RNA present in their peripheral blood; it occurs rarely if the maternal viral load less than 1×10^5 HCV RNA copies/mL of plasma[82].

Two previous studies demonstrated that high levels of ALT in the year before pregnancy are linked with a higher maternal-fetal transmission rate. These results suggest that the development of liver damage in the mother is a potential risk factor for HCV transmission[83]. Furthermore, HCV infection and signs of viral replications in maternal peripheral blood mononuclear cells enhances the rate of transmission[84]. Conversely, breastfeeding and genotype do not appear to be linked to MTCT. A co-infection with HIV virus increases the likelihood of vertical HCV transmission by 90%[85].

The standard treatment for chronic HCV infection is PEG-IFNα and ribavirin. Recently, the new antiviral medicines, telaprevir and boceprevir were introduced[86].

Little is known about the real impact of gender on the characteristics that influence the efficacy and safety of chronic hepatitis C treatment. Several studies have demonstrated that the sustained virological response (SVR) rate is significantly higher in women than in men, and fertile women with normal genotypes have a 100% chance of obtaining a SVR. Therefore the administration of combined therapy is not recommended during pregnancy (Pregnancy FDA Category X)[87] (Table 5).

GENETIC HEMOCHROMATOSIS

Iron is essential for many biological processes. The liver stores for iron and plays a central role in the regulation of iron metabolism. The liver synthesizes hepcidin, which is the most important iron regulatory hormone.

Genetic hemochromatosis (GH) is a prevalent iron overload disorder in the Caucasian population. Patients absorb more than the normal amount of iron through the intestine. Hepcidin is suggested to play a role in GH.

GH is not a gender-specific disease, but more males than females present symptoms and signs of hemochromatosis. Men accumulate more iron and have a higher incidence of liver injury[88].

The clinical symptoms of GH usually begin later in women than in men, likely due to the physiological loss of blood in women of childbearing age. The gender-specific regulation of hepcidin synthesis in the liver may play a role in this process[89].

The prevalence of the disease in men may also be explained by the greater extrahepatic deposition of iron in males than in females. In addition, serum ferritin levels are higher in men, which suggests that men have increased extrahepatic iron stores[90].

In conclusion, the clinical presentation of GH is different between women and men. Both liver disease and diabetes are more common in men, whereas fatigue and pigmentation are more common in women[91].

CONCLUSION

Gender medicine focuses the scientific community on understanding and analyzing clinical, patho-physiological, prevention and treatment differences in diseases that are equally represented in men and women[1].

Current medicine offers better care through the study of disease mechanisms based on gender differences by focusing on the incidence and etiology of pathologies, clinical objectives and the response to therapies[33]. The purpose of this fields is to provide the best treatment possible to each individual man and woman based on scientific evidences.

This review emphasized the importance of appropriate management of viral chronic hepatitis during pregnancy and summarized the strategies to prevent mother-to-child transmission. The review focused on maternal and perinatal outcomes, disease progression and its impact on pregnancy, and the new effective drugs used to

prevent maternal infection transmission without significant adverse effects or complications. In summary, based on the current literature, we recommend close maternal-fetal monitoring during pregnancy and suggest that all available treatment options be considered in the future.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (10): Alcoholic liver disease

Role of mitochondria in alcoholic liver disease

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Abstract

Alcohol abuse is the leading cause of liver related morbidity and mortality. Chronic or binge alcohol drinking causes hepatic steatosis which can develop to steatohepatitis, cirrhosis and ultimately hepatocellular carcinoma. The pathogenesis of alcoholic liver disease (ALD) is poorly characterized, however several recent studies point to a major role of mitochondria in this process. Mitochondria play a crucial role in cellular energy metabolism and in reactive species formation. Alcohol treatment causes mitochondrial DNA damage, lipid accumulation and oxidative stress. Studies in both animal models and in humans showed that alcohol administration causes changes in the mitochondrial morphology and function suggesting a role of these changes in the pathogenesis of ALD. We review recent findings on mechanisms by which alcohol negatively impacts mitochondrial biogenesis and function and we will discuss the specific intracellular pathways affected by alcohol consumption. Interestingly, recent findings indicate that a large number of mitochondrial proteins are acetylated and that mitochondrial proteins acetylation and sirtuins are modulated by alcohol. Understanding the mechanisms behind alcohol mediated impaired mitochondrial biogenesis and function may help identify potential therapeutic targets for treating ALD in humans.

Key words: Liver; Alcoholic liver disease; Mitochondria; Alcohol; Sirtuins

Core tip: Excessive chronic or binge alcohol consumption causes alcoholic liver disease (ALD) with a spectrum ranging from simple steatosis to steatohepatitis and cirrhosis. One of the characteristics of ALD is the alteration in mitochondrial structure and function. This review summarizes some of the recent findings of the molecular mechanisms involved in the modulation of mitochondrial function and their implication in the development of ALD.

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INTRODUCTION

Alcohol is widely consumed in most parts of the world and has long been associated with various liver diseases accounting for about 4% of all deaths[1]. The 2011 global status report on alcohol and health (World Health Organization) indicated that 4.5% of the global burden of disease and injury was attributed to alcohol with 7.4% for men and 1.4% for women. In the United States, 50% of the adult population (aged 18 years and over) consumed alcohol on regular basis in 2011 (Summary Health Statistics for United States Adults: National Health Interview Survey, 2011). Excessive chronic or

binge (acute large doses) of alcohol consumption causes hepatic steatosis, which can progress, if drinking continues, to more advanced form of alcoholic liver disease (ALD) such as alcoholic steatohepatitis (SH), hepatic fibrosis and cirrhosis and ultimately hepatocellular carcinoma[2-4]. The pathogenesis of ALD is still poorly understood making the progress in finding treatment slow. One of the characteristics of ALD both in animal models and in patients is the perturbation in the morphology and function of mitochondria. Abnormal mitochondrial and cellular redox homeostasis has been documented in alcoholic steatohepatitis and results in alterations of multiple redox-sensitive signaling cascades[5]. We will review current understandings of the role of alcohol metabolism in the pathogenesis of liver disease and the recent mechanisms involved in ALD with special focus on the mitochondrial changes associated with alcohol consumption and their potential implications in ALD.

SPECTRUM OF ALCOHOLIC LIVER DISEASE

ALD is a multistage disease consisting of hepatic steatosis (fatty liver), alcoholic hepatitis, and chronic hepatitis (inflammation) with hepatic fibrosis (development of scar tissue) or cirrhosis[5,6]. The different stages of ALD are not mutually exclusive and may be present simultaneously in certain individual[7]; alcoholic cirrhosis can develop without precedent of hepatic steatosis or alcoholic hepatitis. Hepatic steatosis is the earliest response to alcohol consumption and develops in 90% of heavy alcohol drinkers[8-10]. Simple hepatic steatosis is usually asymptomatic, reversible and resolve after 4-6 wk of abstinence[11]. With continuous alcohol intake, 20%-30% of patients with steatosis develop alcoholic hepatitis and 16% of patients with steatohepatitis will develop cirrhosis[12,13]. Fibrosis of the liver, a consequence of inflammation, infection or injury, results from excessive accumulation of collagen and other extracellular matrix proteins in the liver which impedes normal function of the liver leading to the development of cirrhosis. Alcohol associated cirrhosis is a consequence of sustained alcohol intake and is characterized by both steatosis and hepatitis with fibrosis[4]. Alcoholic cirrhosis is irreversible and is among the top ten causes of death worldwide. According to the National Institute on Alcohol Abuse and Alcoholism, liver cirrhosis is the 12th leading cause of death in the United States with 29925 death in 2010, about 50% of which are alcohol related[14]. Liver cirrhosis predisposes the HCC, it is seen in about 80% of HCC patients[15,16]. In the US, HCC is the most rapidly growing cause of cancer-related mortality, particularly among men ages 40 to 60 years[17,18].

Although hepatic steatosis is found in 90% of heavy alcohol drinkers, the severe forms of ALD such as fibrosis and cirrhosis develop only in 30% of individuals with heavy alcohol intake suggesting that other factors are involved in the progression of the disease. The possible risk factors that can affect the development of liver injury include the dose[19], duration[20], type of alcohol consumed[21], drinking patterns[22], gender[4,23], ethnicity[4,24], and associated risk factors, including obesity[25,26] and genetic factors[27,28].

METABOLISM OF ALCOHOL IN THE LIVER

When consumed, 90% of ingested alcohol is absorbed in the upper GI tract and diffuse throughout the body[29,30]. Studies both in humans[31,32] and animals[33-35] have shown that both short and long term ethanol treatment can disrupt the epithelial barrier of the GI tract which results in increased intestinal permeability and enhanced movement of luminal antigens such as bacteria and endotoxins into the portal circulation[36,37]. This can lead to Kupffer cells activation in the liver and cytokine release which may consequently results in liver injury and ALD[38,39]. The liver is the main organ responsible for metabolizing ingested alcohol; therefore it is more susceptible to alcohol related injury. Alcohol is metabolized in the liver by both oxidative and non-oxidative pathways. Briefly, the oxidative pathways of alcohol metabolism involve three enzymes, alcohol dehydrogenase (ADH) in the cytosol, cytochrome P450 2E1 (CYP2E1) in the peroxisomes and catalase in the microsomes[29,40]. ADH is present in the cytosol where it converts alcohol to acetaldehyde and other metabolites. In this reaction nicotinamide adenine dinucleotide (NAD^+) is reduced by two electrons to NADH generating a highly reduced environment in hepatocytes. The increased NADH/NAD ratio favors hepatic triglyceride accumulation. In addition, excess NADH may promote fatty acid synthesis. Acetaldehyde inhibits protein synthesis and may be linked to tumor development[41]. Cytochrome P450 enzymes (Cyp2E1, 1A2, and 3A4) present mainly in microsomes and endoplasmic reticulum also contribute to alcohol metabolism into acetaldehyde at high concentration of ethanol. Catalase in the peroxisomal pathway requires hydrogen peroxidase (H_2O_2) to oxidize alcohol into acetaldehyde and water. Acetaldehyde is a highly reactive and toxic byproduct to hepatocytes that may contribute to tissue damage because it forms a variety of protein and DNA adducts that promote glutathione depletion, lipid peroxidation, and mitochondrial damage[40,41]. It also contributes to the changes in the redox state of the cell and the formation of reactive oxygen species (ROS)[41]. The acetaldehyde produced from alcohol oxidation is rapidly metabolized into NADH and acetate by aldehyde dehydrogenase (ALDH) in mitochondria. The product of acetaldehyde breakdown is rapidly removed from the liver and is metabolized into CO_2 *via* the TCA cycle in the heart, skeletal muscle and the brain. Genetic variations in ADH and ALDH influence the susceptibility of developing alcoholism and alcohol related injury. The nonoxidative pathway is minor in normal conditions and leads to the formation of fatty acid ethyl ester

(FAEE) and phosphatidyl ethanol (PEth). The products of the nonoxidative pathway have pathological and diagnostic significance. Both PEth and FAEE are poorly metabolized; they accumulate in the liver and interfere with cell signaling. Because of their intermediate half-life and tendency to accumulate, non-oxidative ethanol metabolites can be used as biomarkers for alcohol consumption[42]. A second nonoxidative pathway occurs at high circulating levels of alcohol and involves phospholipase D (PLD), which converts phosphatidylcholine to generate phosphatidic acid (PA) and subsequently phosphatidyl ethanol[40]. Phosphatidyl ethanol is poorly metabolized and its effects on the cell are unknown, however it might interfere with the production of PA and disrupt cell signaling[40].

ROLE OF MITOCHONDRIA IN LIVER PHYSIOLOGY

Mitochondrial structure

Mitochondria are organelles with double membrane structure. The outer membrane delimits the intermediate space while the inner membrane delimits the mitochondrial matrix. The structure of the inner membrane is highly complex and consists of the complexes of the electron transport system, the ATP synthetase complex, and transport proteins. The matrix contains a highly concentrated mixture of enzymes involved in the oxidation of pyruvate and fatty acids (FAs) in TCA cycle. Eukaryotic cells contain nuclear and mitochondrial DNA (mtDNA) genomes sequestered into distinct subcellular compartments. Human mtDNA is found in the matrix and consists of 13 structural genes that encode subunits essential for respiratory complexes Ⅰ, Ⅲ, Ⅳ, and Ⅴ of the mitochondrial respiratory chain involved in the generation of ATP. Each mitochondrion contains 5-10 copies of mtDNA and each cell contains a high copy number of mtDNA. It is considered that the number of normal mtDNA copies must fall below 20%-40% of basal levels to induce mitochondrial dysfunction and the adverse effects. The mitochondrial matrix possesses an incomplete mitochondrial DNA repair system, and is highly sensitive to ROS-induced oxidative damage due to its proximity to the inner mitochondrial membrane where most of the ROS are produced[43-45].

Mitochondrial function

Mitochondria are the power producer of the cell which plays a central role in the generation of energy from nutrient oxidation. Hepatocytes are rich in mitochondria; each hepatocyte contains about 800 mitochondria occupying about 18% of the entire cell volume. Mitochondria have a unique role in the liver compared to other organs as they participate in glucose, lipids and protein metabolism. Mitochondria play an essential role in the cell as they provide the majority of cellular energy in the form of ATP; generate and regulate ROS; buffer cytosolic Ca^{2+} and regulate apoptosis. Carbohydrates and fats are oxidized in the mitochondria to produce energy. Glucose is converted into pyruvate within the cytosol, transported to the mitochondria where it is converted to acetyl-CoA. Acetyl-CoA undergoes TCA cycle resulting in CO_2, water and energy. FAs in the liver originate from the diet, adipose tissue lipolysis, or hydrolysis of intracellular stores or *de novo* lipogenesis. These free FAs (FFAs) are metabolized through oxidation, ketogenesis or esterification into triglyceride. Together with the muscle, the liver is the main site for mitochondrial fatty acid oxidation. The oxidation of FFAs occurs mainly in the mitochondria of hepatocytes where FFAs are converted by carnitine palmitoyltransferase-1 (CPT1) into acylcarnitine which is transported into the mitochondrial matrix. FFAs oxidation into acetyl-coenzyme A (acetyl-CoA) and its subsequent oxidation by the TCA cycle generates reduced NADH and reduced flavine-adenin dinucleotide ($FADH_2$). NADH and $FADH_2$ are the electron donor and transfer the hydrogen/electron to an oxygen molecule, *via* a variety of redox components in complex Ⅰ through Ⅳ in the mitochondrial respiratory chain located in the inner mitochondrial membrane[46]. The flow of electron in the respiratory chain is coupled with pumping of protons from the mitochondrial matrix into the inter-membrane space, thus creating an electrochemical gradient across the membrane[46]. When ATP is low, protons re-enter the matrix trough ATP synthase and the energy released initiates ATP synthesis[46]. Although most of the electrons end up in water, the insulation of complexes Ⅰ and Ⅲ of the respiratory chain from oxygen in not perfect[46]. Excessive electron flow to the respiratory chain results in accumulation and leakage of electrons at these two sites which react with oxygen to produce ROS[47]. About 1%-2% of mitochondrial oxygen consumption results in ROS production under normal condition[48], 90% of cellular ROS are produced in the mitochondria[49]. Mitochondria play an important role in ROS homeostasis as they are the main site for ROS generation but can also be a target for excessive ROS exposure. The ROS production occurs through reduction of oxygen to superoxide (O^{2-}) by complex Ⅰ and Ⅲ of the electron transfer chain (ETC). Under normal conditions Mn-SOD located in the matrix of mitochondria converts the O^{2-} to H_2O_2 which is subsequently reduced to H_2O. ROS production in mitochondria is upregulated in conditions where increased NADH and increased membrane potential is not coupled with an increase in ATP production. Excessive production of ROS exceeding the cell's antioxidant defenses can damage components of the cells such as lipids, proteins and nucleic acids (particularly mtDNA) leading to oxidative stress and ultimately apoptosis. This can be observed in conditions of increased oxidation of FFAs such as in NASH[50] and when levels of NADH are augmented due to alcohol metabolism in alcoholic steatohepatitis[45].

MITOCHONDRIAL ALTERATION IN ALD

Recent studies have suggested that chronic ethanol administration causes changes in the mitochondrial morphology and function in both animal models and humans. The mitochondria are often enlarged and altered and these structural changes are associated with the development of fatty liver in the rat[51] suggesting that chronic ethanol treatment affects hepatic energy metabolism. With the exception of one study that showed an increase in mitochondrial respiration in mice[52], most studies in rats and in humans have shown altered mitochondrial respiration. The mechanism behind the species difference between the rat and mouse is unknown. Hepatocytes isolated from mice fed ethanol containing diet showed lower fatty acid oxidation and increased lipid synthesis. In the rat, chronic ethanol administration alters mitochondrial oxidative phosphorylation in the liver by inhibiting the synthesis of proteins of the respiratory complexes[53]. Lower oxidation capacity in combination of the reducing environment induced by ethanol creates conditions of O^{2-} formation and ROS production. As mentioned above, mtDNA, which encodes the subunits of the electron transport chain and the ATP synthase, is vulnerable to ROS due its proximity to the source (the inner membrane) of cellular ROS. Damage of mtDNA will in turn impair cellular energy metabolism and enhances ROS formation. Strong evidence indicates that oxidative stress and dysregulation of redox-sensitive signaling pathways are central to the pathobiology of ALD. It has been shown that a single dose of ethanol was able to damage mtDNA and cause cell toxicity[45]. Other abnormalities that have been described as a result of ethanol treatment are decreases in ATP levels. Oxidative stress can cause cellular apoptosis *via* both mitochondria-independent (involving death receptor of the tumor necrosis factor (TNF) receptor gene family) and mitochondria-dependent (caused by intracellular stresses such DNA damage and ROS) pathways. Hepatocyte apoptosis has been observed in patients with alcoholic hepatitis[54]. One of the mechanisms proposed to explain alcohol-induced hepatocyte apoptosis is the release of cytochrome C in the cytosol where it promotes caspases activation. The role of alcohol in ROS production, the induction of the mitochondrial cell death pathway and the possible mechanisms involved have been recently discussed in[55].

Acetylation is an important posttranslational modification that regulates proteins. Recently, sirtuins, a family of NAD^+ dependent deacetylases have been identified. In mammals, sirtuins are a family of seven proteins (SIRT1-7) that have been shown to be involved in longevity, DNA repair and the control of metabolic enzymes. Three sirtuins SITR3, SIRT4 and SIRT5 are localized within the mitochondrial matrix[56]. Interestingly, SIRT3-deficient mice show increased mitochondrial protein hyperacetylation but not SIRT4 and SIRT5 deficient mice suggesting that SIRT3 is a major mitochondrial deacetylase[57,58]. Chronic alcohol consumption induces a global acetylation of proteins mainly mitochondrial proteins indicating a role of the acetylation of mitochondrial proteins in mitochondrial biology. At least 20% of mitochondrial proteins are acetylated including proteins of TCA cycle, oxidative phosphorylation, β-oxidation and the urea cycle[59]. Therefore, Sirtuins have been implicated in the regulation of mitochondrial number, turnover and activity and have been proposed to play a role in the pathogenesis of alcoholic liver disease[60]. Chronic ethanol administration impairs hepatic lipid metabolism pathways largely by modulating SIRT1 (a nuclear sirtuin) and causes development of fatty liver. Ethanol decreases hepatic SIRT1 in rodent models suggesting a role of ethanol and SIRT1 in the regulation of mitochondrial energy metabolism and mitochondrial biogenesis. SIRT1 regulates lipid metabolism by deacetylating the sterol regulatory element-binding protein-1c (SREB-1c) and PPARγ coactivator 1α (PGC-1α); thus it increases fatty acid synthesis and decreases fatty acid β-oxidation (deacetylation of PGC-1α increases its activity; deacetylation of SREBP-1c decreases its activity). In addition, evidence suggests that mitochondrial biogenesis is regulated at least in part by PGC-1α suggesting that ethanol mediated reduced deacetylation of PGC-1α would inhibit mitochondrial biogenesis[61]. More recent studies, have demonstrated that chronic alcohol treatment specifically induced a small noncoding micro RNA (miRNA) (miR-217) in AML-12 hepatocytes and in mouse livers[62]. Ethanol induced expression of miR-217 reduced SIRT1 expression which in turn results in an increase in lipogenic enzymes [acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), and stearoyl-coenzyme A desaturase 1 (SCD1)] and a decrease in genes involved in fatty acid oxidation [PPARα, PGC-1α, and acyl-CoA oxidase (*AOX*)][60,62]. In addition miR-217 modulation of SIRT1 has been recently shown to regulate lipin-1 which is a crucial regulator of hepatic lipid metabolism[62]. Lipin-1 deficiency aggravated the defect in fatty acid oxidation and lipoprotein secretion induced by alcohol in mice fed the modified Lieber-DeCarli ethanol-containing low-fat diets for 4 wk[62] suggesting a role of this microRNA in the development of ALD. It is not known how alcohol induces miR-217 but it is suggested that the products of ethanol metabolism, acetaldehyde and acetate, may play a role in this process[62].

Recent studies suggest that sirtuins can modulate ROS levels. As mentioned above, mitochondria are involved in the generation of ROS as well as in the defense against ROS. In addition, mitochondria are themselves target for ROS damage and cell fate. Mitochondrial SIRT3 deacetylates and activates enzymes involved in maintaining physiological ROS levels. SIRT3 reduces ROS species levels through deacetylation and activation of the antioxidant enzyme superoxide dismutase (SOD)[63,64]. SIRT1 is essential for ROS-mediated apoptosis in embryonic stem cells by facilitating mitochondrial localization of p53[65]. SIRT1 also inactivates the P65 subunit of NF-κB through direct acetylation, NF-κB inhibition suppresses the inducible nitric oxide synthase

(iNOS) and nitrous acid production and thus may lower cellular ROS levels[66].

Together the recent findings point to a major role of mitochondria in alcohol induced hepatic fat accumulation. Alcohol consumption results in inhibition of PPAR-α and stimulation of SREBP-1C and the transformation of liver from an oxidizing to a fat-storing organ. Alcohol also induces reactive oxygen species levels and causes liver injury and apoptosis in part by regulating sirtuins levels and enzymes of the antioxidant defense.

CONCLUSION

Alcohol has deleterious effects on liver function. Ethanol and its metabolites destroy the liver over time. Mitochondria play an important role both in hepatic alcohol metabolism and in the bioenergetics of the hepatocyte. In this review we have outline the mechanisms by which alcohol negatively impacts mitochondrial function. The observed changes in mitochondrial function and the alteration in redox status and their implication in alcohol induced hepatic steatosis and the progression of liver disease have been discussed. We also reviewed some of the recent pathways involved in the development of ALD. Future research into the mechanism by which alcohol modulates mitochondrial biogenesis and function are critical for the development of biomarkers and the identification of potential therapeutic targets for the prevention and treatment of human ALD.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (10): Alcoholic liver disease

Therapy for alcoholic liver disease

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Abstract

Alcoholism results in about 2.5 million deaths annually worldwide, representing 4% of all mortality. Although alcoholism is associated with more than 60 diseases, most mortality from alcoholism results from alcoholic liver disease (ALD). ALD includes alcoholic steatosis, alcoholic hepatitis, and alcoholic cirrhosis, in order of increasing severity. Important scoring systems of ALD severity include: Child-Pugh, a semi-quantitative scoring system useful to roughly characterize clinical severity; model for end-stage liver disease, a quantitative, objective scoring system used for prognostication and prioritization for liver transplantation; and discriminant function, used to determine whether to administer corticosteroids for alcoholic hepatitis. Abstinence is the cornerstone of ALD therapy. Psychotherapies, including twelve-step facilitation therapy, cognitive-behavioral therapy, and motivational enhancement therapy, help support abstinence. Disulfiram decreases alcohol consumption by causing unpleasant sensations after drinking alcohol from accumulation of acetaldehyde in serum, but disulfiram can be hepatotoxic. Adjunctive pharmacotherapies to reduce alcohol consumption include naltrexone, acamprosate, and baclofen. Nutritional therapy helps reverse muscle wasting, weight loss, vitamin deficiencies, and trace element deficiencies associated with ALD. Although reduced protein intake was previously recommended for advanced ALD to prevent hepatic encephalopathy, a diet containing 1.2-1.5 g of protein/kg per day is currently recommended to prevent muscle wasting. Corticosteroids are first-line therapy for severe alcoholic hepatitis (discriminant function $\geqslant$ 32), but proof of their efficacy in decreasing mortality remains elusive. Pentoxifylline is an alternative therapy. Complications of advanced ALD include ascites, spontaneous bacterial peritonitis, esophageal variceal bleeding, hepatic encephalopathy, hepatorenal syndrome, hepatopulmonary syndrome, and portopulmonary hypertension. Alcoholic cirrhotics have increased risk of developing hepatomas. Liver transplantation is the ultimate therapy for severe ALD, but generally requires 6 mo of proven abstinence for eligibility. Alcoholic cirrhotics who maintain abstinence generally have a relatively favorable prognosis after liver transplantation.

Key words: Alcoholic liver disease; Alcoholic steatosis; Alcoholic hepatitis; Alcoholic cirrhosis; Alcoholism; Liver disease; Corticosteroids; Pentoxifylline; Liver transplantation

Core tip: Alcoholism results in about 2.5 million deaths annually worldwide, representing 4% of all mortality. Most of this mortality is from alcoholic liver disease (ALD). ALD includes alcoholic steatosis, alcoholic hepatitis, and alcoholic cirrhosis, in order of increasing severity. This work reviews this clinically important subject, with a focus on informing clinicians of recent advances in therapy to reduce the currently high mortality from alcoholic hepatitis and alcoholic cirrhosis.

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INTRODUCTION

Alcoholism results in an estimated 2.5 million deaths annually worldwide, representing 4% of all mortality[1]. This mortality is much greater than that caused by acquired immunodeficiency syndrome (AIDS) or tuberculosis[1]. It is the leading risk factor for mortality for ages 15-59 in males, and the eighth leading risk factor for mortality for all ages in both sexes[1]. Alcoholism is associated with more than 60 diseases, and is commonly associated with accidental injuries, including motor vehicle injuries[1]. Alcoholic liver disease (ALD), moreover, accounts for 40% of mortality from cirrhosis[2]. Annual mortality for ALD is 4.4 per 100000 in the general population, compared to 2.9 per 100000 for hepatitis C virus (HCV)[3].

Alcoholism, or alcohol use disorder, is defined, according to the *Diagnostic and Statistical Manual of Mental Disorders*, 5th edition (DSM-5), as a problematic pattern of alcohol use leading to clinically significant impairment or psychological distress[4]. Development of ALD is dose-dependent, and drinking ≥ 30 g/d of alcohol ("standard" drink: contains 0.6 fluid ounces or 14 g "pure" alcohol[5] increases the risk of ALD in both sexes[6]). Women have a greater risk of ALD than men, likely secondary to differences in ethanol metabolism[7-13]. For example, one study reported the threshold level of alcohol intake for developing ALD is 12-22 g/d in women *vs* 24-46 g/d in men[7]. Many alcoholic patients, however, do not develop clinically significant ALD[14]. Genetic and environmental factors are important, but the specific genes or environmental factors that predispose to ALD are poorly understood[14]. Potentiating factors for ALD include metabolic syndrome[13,15], diabetes[16,17], obesity[17,18], smoking[13,15], iron overload[12,13,17,19], and chronic viral hepatitis B or C[12,13,20,21].

The only definitive treatment for ALD is liver transplantation (LT). Abstinence is critical, but usually cannot reverse advanced ALD. Supportive therapy and nutritional management are also important. Several medical therapies have been studied, including corticosteroids and pentoxifylline, but no medical therapy has been proven to improve survival. This review discusses the spectrum of ALD; reviews the classification of severity and prognostic criteria for ALD; analyzes the current therapies for ALD, including abstinence, nutritional therapy, drugs, and LT; discusses the controversies regarding survival benefits for individual therapies; and reports professional society guidelines for management of cirrhosis and its complications.

SPECTRUM OF ALD

ALD is classified into alcoholic fatty liver (steatosis), alcoholic hepatitis (AH; steatohepatitis), and alcoholic cirrhosis. About 90% of alcoholics develop alcoholic steatosis, about 25% develop alcoholic hepatitis, about 15% develop alcoholic cirrhosis, and about 10% develop hepatocellular carcinoma[13,22-24]. Alcoholic steatosis, the earliest manifestation of ALD, is pathologically characterized by microvesicular and macrovesicular fat accumulation within hepatocytes, minimal inflammatory reaction, and no hepatic fibrosis[23]. It is often reversible with abstinence[25]. Patients are often asymptomatic, and the diagnosis is usually incidental. They do not exhibit stigmata of chronic liver disease, such as spider angiomata and palmar erythema. Patients typically present with mild elevations of liver enzymes, including gamma-glutamyl transpeptidase (GGT), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels[25]. The serum bilirubin level and liver synthetic function [international normalized ratio (INR), albumin level] tend to be normal.

AH is an inflammatory process with predominantly neutrophilic infiltration, characterized by ballooning degeneration of hepatocytes, hepatocyte necrosis, steatosis, and presence of Mallory bodies (homogeneous, eosinophilic cytoplasmic perinuclear inclusions) within hepatocytes[23] (Figure 1A). Clinical findings include jaundice, pyrexia, unintentional weight loss, malnutrition, and tender, enlarged liver[26]. Patients typically present with moderate elevations in AST (usually < 300 IU/L), ALT, GGT, and serum bilirubin[25]. An AST:ALT ratio of ≥ 2:1 is strongly suggestive of ALD[27], likely from up-regulated mitochondrial AST molecular expression and synthesis by alcohol[28]. AH may be complicated by ascites, encephalopathy, or gastrointestinal (GI) bleeding from esophageal varices or portal gastropathy[26]. Imaging may show hepatomegaly, but this finding is nonspecific, and imaging is generally performed to identify radiologic evidence of cirrhosis, to identify its complications, and to exclude focal hepatic lesions[25,29]. Definitive diagnosis is by liver biopsy, but this is rarely necessary in clinical practice.

Alcoholic cirrhosis is pathologically characterized by severely disorganized liver architecture, with both bridging fibrosis and regenerating nodules, that are typically uniformly-sized and micronodular[23] (Figure 1B). Patients usually present with stigmata of chronic liver disease, including gynecomastia, palmar erythema, spider angiomata, testicular atrophy, and parotid gland enlargement; and signs of portal hypertension, including caput medusa. Dupuytren's contracture is often present in patients with alcoholic cirrhosis[30]. Alcoholic cirrhosis is associated with multiple complications, as discussed below. Laboratory findings often include hypoalbuminemia, hyperbilirubinemia, thrombocytopenia, and prolonged prothrombin time (PT) and increased INR[26]. These abnormalities typically worsen with progression of cirrhosis. Imaging studies often demonstrate findings consistent with cirrhosis, including a small, shrunken liver, hepatic nodularity, abnormal tortuous vessels from intra-abdominal varices, and other abnormalities, such as ascites or focal hepatic lesions[25,31]. These findings assist in diagnosing cirrhosis,

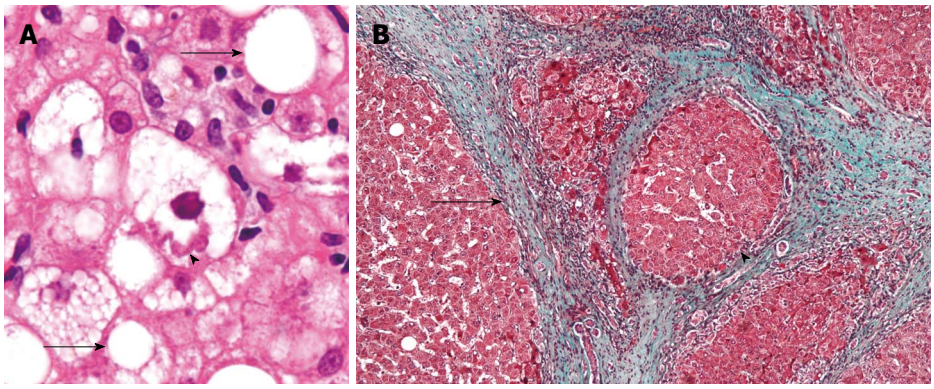

Figure 1 Photomicrograph. A: Photomicrograph showing a Mallory body (arrowhead), with twisted rope-like appearance, and fat vacuoles (arrows) as seen in alcoholic steatohepatitis (image from http://en.wikipedia.org/wiki/Alcoholic_hepatitis, licensed under Creative Commons Attribution-Share Alike 3.0 Unported); B: Photomicrograph showing a regenerating nodule (arrowhead) and bridging fibrosis (arrow) as seen in alcoholic cirrhosis (image from http://en.wikipedia.org/wiki/Alcoholic_cirrhosis, licensed under Creative Commons Attribution-Share Alike 3.0 Unported).

but cannot, by themselves, establish alcohol as the etiology[26,32]. Magnetic resonance imaging (MRI) findings suggestive of alcoholic cirrhosis include caudate lobe enlargement, presence of the right posterior hepatic notch, and smaller regenerative nodules (micronodular) than in other etiologies of cirrhosis[31]. Alcoholic cirrhosis is diagnosed by history of excessive alcohol intake, with exclusion of other causes of cirrhosis (Table 1)[33-36]. The evolution of alcoholic fatty liver to alcoholic hepatitis to alcoholic cirrhosis is usually progressive with continued alcohol use, but different stages can occur simultaneously in one patient (*e.g.*, AH with alcoholic cirrhosis).

PROGNOSTIC CRITERIA AND MONITORS OF DISEASE SEVERITY

Several scoring systems assess severity of liver disease and predict patient survival. Child-Turcotte-Pugh (CTP) score, the oldest scoring system, uses serum bilirubin level, albumin level, PT, severity of ascites, and severity of encephalopathy[37]. Patients are categorized as: class A = scores 1-6, class B = scores 7-9, and class C = scores 10-15; the higher the score, the worse the disease[37,38]. Although heuristically useful, CTP scores are limited by subjectivity in grading and by use of PT instead of the more accurate INR[39]. It was previously used to prioritize candidates for LT, but was supplanted in 2002 by the more quantitative and less subjective model for end-stage liver disease (MELD) score[40]. MELD score was originally developed to assess short-term prognosis in cirrhotic patients undergoing transjugular intrahepatic portosystemic shunt (TIPS), but was found to also reliably estimate short-term survival in patients with any type of chronic liver disease[41-43]. MELD score includes serum bilirubin level, creatinine level, and INR[42]. It can be calculated on-line at a free website[44]. The United Network for Organ Sharing (UNOS) uses MELD score to prioritize LT candidates because it relatively accurately predicts 3-mo mortality in patients awaiting LT[39,40]. Recently, addition of serum sodium concentration (MELD-Na score) has been proposed to more accurately predict mortality in patients awaiting transplant[45,46]. The MELD-Na score, if used for liver allocation, can avert an additional 7% mortality in patients awaiting LT[45]. It is not yet widely used, but is a promising scoring system that may better predict mortality and improve donor liver allocation.

While CTP and MELD scores are applicable to all etiologies of cirrhosis, other scoring systems are specific for ALD. Discriminant function (DF), which includes only PT and serum bilirubin, is used to predict early mortality in AH patients and to objectively select AH patients likely to benefit from corticosteroid therapy[47,48]. DF < 32 is classified as non-severe AH, with 10% mortality, whereas DF $\geqslant$ 32 is classified as severe AH, with mortality ranging from 30%-60% without treatment[48-55]. Glasgow alcoholic hepatitis score (GAHS) is calculated as the sum of scores for the following individual parameters: age, leukocyte count, serum urea level, PT ratio (ratio of patient-to-control PT), and serum bilirubin level[56]. A score > 8 predicts poor prognosis[56]. ABIC, the most recent scoring system, includes age, serum bilirubin, INR, and serum creatinine[57]. It stratifies risk of mortality from AH as low (score < 6.71), intermediate (score: 6.71-8.99), and high (score $\geqslant$ 9.0), with 90 d mortality at 0%, 30%, and 75%, respectively ($P < 0.0001$)[57]. These last two scoring systems are promising prognostic indicators and treatment guides, but are currently rarely used clinically.

Lille score includes 6 variables: age, albumin level, bilirubin level at day 0, bilirubin level at day 7, PT, and presence of renal insufficiency[58]. It helps stratify patients with AH. It may be more accurate than other scoring systems, but is mostly used to predict 6-mo survival in patients with AH treated with corticosteroids[59]. It predicts about 75% of observed 6-mo mortality[59]. A score < 0.45 predicts 15% mortality, whereas a score $\geqslant$ 0.45 predicts 75% mortality ($P < 0.0001$)[59]. Alternative therapies should be considered when the score is $\geqslant$ 0.45 at day 7 of corticosteroid therapy[59].

Table 1 Differential diagnosis of cirrhosis, excluding alcoholic cirrhosis[1]

Diseases	Diagnostic studies	Liver biopsy
Wilson's disease	Serum ceruloplasmin < 20 mg/dL; 24-h urine copper excretion > 100 μg/24 h; slit-lamp ophthalmologic examination for Kayser-Fleischer rings	Steatosis; glycogenated nuclei in hepatocytes; focal hepatocellular necrosis, fibrosis, and ultimately, cirrhosis, usually macronodular[34]; copper retention in hepatocytes; hepatic copper concentration > 250 μg/g dry weight
Hemochromatosis	Serum transferrin-iron saturation > 45%; serum ferritin typically > 1000 μg/L; genotyping for detection of *HFE* mutations: C282Y and H63D; non-contrast CT of liver demonstrates attenuation values of > 70 HU[36]	Grade 4 stainable iron in hepatocytes, with periportal distribution and sparing of Kupffer cells; hepatic iron concentration > 80 μmol/g dry weight; hepatic iron index > 1.9
Hepatitis C	Anti-HCV; HCV RNA in patients who test positive for HCV antibody	Triad of histological findings with acute infection: lymphoid aggregates in portal tracts, epithelial damage of small bile ducts, and prominent microvesicular and macrovesicular steatosis; chronic infection: periportal necrosis, intralobular necrosis, portal inflammation, and fibrosis; no characteristic pathognomonic features
Chronic hepatitis B	HBsAg; serum level of HBV DNA > 2000-20000 IU/mL	Acute infection: lobular disarray, ballooning degeneration, numerous apoptotic (Councilman) bodies, Kupffer cell activation, and lymphocyte-predominant lobular and portal inflammation; chronic infection: varying degree of predominantly lymphocytic portal inflammation with interface hepatitis and spotty lobular inflammation[34]; presence of HBcAg staining in the liver
Autoimmune hepatitis	Antinuclear antibody (ANA); smooth muscle antibody (SMA); antibodies to liver and kidney microsomes (anti-LKM1); anti-soluble liver antigen (anti-SLA); asialoglycoprotein receptor antibodies	Interface hepatitis at junction of portal region and liver lobule; lobular hepatitis with lymphocytoplasmacytic infiltration; intrahepatic bile ducts generally appear normal
α1-antitrypsin deficiency	Serum α1-antitrypsin genotype or phenotype (homozygous PiZZ or heterozygous PiSZ phenotype)	Giant-cell hepatitis with multinucleated giant cells; lobular disarray; cellular and canalicular cholestasis; neoductular proliferation; bridging hepatic fibrosis; PAS-positive and diastase-resistant cytoplasmic granules in periportal hepatocytes
Primary biliary cirrhosis	Antimitochondrial antibodies (AMA) ⩾ 1: 80 titer; ANA, with immunofluorescence typically revealing speckled, homogeneous, nuclear dot, centromere, or rim-like patterns	Focal and segmental nonsuppurative cholangitis; "florid duct lesion": bile duct surrounded by intense lymphocytic or granulomatous infiltrate with basal integrity of the bile duct breached by individual lymphocytes; granulomas in close proximity to bile duct; bile ductular proliferation (cholangioles or pseudoducts) along periphery of portal tract
Non alcoholic fatty liver disease	Diagnosis of exclusion, correlated with: metabolic syndrome: diabetes mellitus, hypertension, hyperlipidemia, abdominal obesity with waist circumference > 102 cm for men and > 88 cm for women; obesity (BMI ⩾ 30 kg/m^2); obstructive sleep apnea; sedentary lifestyle	Macrovesicular steatosis; early hepatocyte inflammation, predominantly neutrophilic; late nondescript fibrosis and cirrhosis

[1]Patients may have more than one disease contributing to cirrhosis (*e.g.*, alcoholism and iron overload, or alcoholism and hepatitis C). *HFE*: Human hemochromatosis protein; CT: Computed tomography; HU: Hounsfield units; HCV: Hepatitis C virus; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; DNA: Deoxyribonucleic acid; HBcAg: Hepatitis B core antigen; PAS: Periodic acid-Schiff; BMI: Body mass index.

THERAPY

Abstinence supportive therapies for abstinence

Abstinence is the cornerstone of therapy. It markedly reduces mortality[60-64]. However, this benefit may not become statistically significant until at least ⩾ 1.5 years of abstinence[65]. Five-year mortality for patients with alcoholic cirrhosis who cease drinking decreases to 10%, compared to 30% for patients who continue to drink[22]. Alcohol consumption increases portal pressure and increases porto-collateral blood flow in cirrhotic patients[66]. Even moderate alcohol consumption significantly worsens hepatic hemodynamics[66]. Abstinence results in a decreased rate of rebleeding after acute variceal bleeding [hazard ratio (HR) 0.26, $P = 0.002$][64]. Abstinence may also decrease hepatic fibrosis, as measured by transient elastography, a noninvasive measure of liver stiffness[67-69]. Liver transplant programs in the United States generally mandate abstinence for ⩾ 6 mo for eligibility for LT[70]. Therefore, strict, long-term abstinence is essential, and physicians should be vigilant for relapses and aggressively intervene in such cases.

Psychotherapies promoting abstinence include twelve-step facilitation therapy (TSF), cognitive-behavioral therapy (CBT), and motivational enhancement therapy (MET)[71]. TSF assumes that alcoholism is a progressive illness for which the only effective remedy is abstinence[72]. It provides a structured program to facilitate active involvement in alcoholics anonymous (AA)[72]. TSF emphasizes the twelve spiritual principles (traditions) of AA, including the first five critical principles focusing on acceptance, surrender, and moral inventories[72]. In "acceptance" patients accept they have a drinking problem. In "surrender" patients accept faith in a "Higher Power" and follow the AA path[72]. CBT identifies "high-risk" situations that increase the risk of alcoholism and encourages patients to assume responsibility and acquire self-control skills to prevent relapse[73]. It consists of 12 sessions to train clients to adopt active behavioral and cognitive methods, rather than alcohol, as coping mecha-

nisms during "high-risk" situations[73]. Lastly, MET employs motivational psychology to produce internally motivated change, *i.e.,* employs strategies to mobilize patient's internal resources for change, rather than continuous external guidance[74]. A large RCT, incorporating 1726 patients, showed all three of these psychotherapies were beneficial and all produced roughly equivalent outcomes[71].

Disulfiram, naltrexone, acamprosate, and baclofen are used to treat alcohol dependence. Disulfiram (Antabuse), an acetaldehyde dehydrogenase inhibitor, alters alcohol metabolism to cause accumulation of serum acetaldehyde, which produces unpleasant sensations of nausea, vomiting, flushing, light-headedness, abdominal pain, and tachycardia[75]. Such unpleasant sensations provide negative reinforcement for ethanol ingestion[75]. However, disulfiram-related hepatotoxicity may result in up to 16% mortality[76], and it must be cautiously administered in patients with ALD. Naltrexone, a mu, kappa, and delta opioid receptor antagonist, decreases alcohol craving by blocking the central pleasurable effects of alcohol. It effectively decreases alcohol intake and prevents relapse[77,78]. A Cochrane meta-analysis reported that naltrexone reduced heavy alcohol consumption by 83%[77]. An extended-release, injectable formulation of naltrexone was reported to improve quality of life, including mental health, social functioning, general health, and physical functioning[79]. It produces relatively mild side effects, mainly nausea, abdominal pain, anorexia, and mild-to-moderate sedation, but is rarely hepatotoxic[77,78,80].

Acamprosate also helps decrease alcohol dependence. It decreases the rate of relapse and helps maintain abstinence[81-85]. Its mechanism of action is unknown[81]. It improves life expectancy and reduces lifetime medical costs in alcoholic patients[86]. It has a favorable safety profile. Diarrhea is the most frequent side effect[81,83]. Baclofen, a gamma-aminobutyric acid-B agonist, is a new, promising, adjuvant treatment for alcohol dependence. It helps alcoholic patients suppress alcohol craving, reduce alcohol intake, and achieve and maintain abstinence[87-90]. It also improves liver function parameters, including serum ALT, bilirubin, GGT, and albumin levels, and INR[88]. These favorable effects occur in alcoholic patients with cirrhosis, with or without concomitant HCV infection[88,90]. Most affirmative studies have been performed in Europe, but a large, randomized controlled trial (RCT) performed in the United States suggested that baclofen is not superior to placebo in treating alcohol dependence[91]. Baclofen produces minimal side effects and has no apparent hepatotoxicity[87-91], rendering it safe in patients with ALD. For all these aforementioned drugs, there are limited data on efficacy in patients with advanced liver disease. Psychotherapy and family support are essential components in the overall management of alcoholics with liver disease.

Nutritional therapy

Muscle wasting, weight loss, and nutritional deficiencies commonly occur in patients with ALD. These abnormalities are associated with increased morbidity and mortality[92,93]. The etiology of weight loss and malnutrition is multifactorial, including poor dietary intake from anorexia, altered sense of taste and smell, and nausea and vomiting; malabsorption; hypermetabolic state; and impaired protein synthesis from cytokine-induced inflammatory responses[92-95]. Nutritional deficiencies can include fat-soluble vitamins (A, D, E and K), folate, thiamine, niacin, and pyridoxine; and the trace elements zinc, magnesium, and selenium[92]. Each deficiency produces specific symptoms, signs, and complications[92]. For example, thiamine deficiency causes Wernicke's encephalopathy.

The European Society for Clinical Nutrition and Metabolism (ESPEN) recommends enteral or parenteral nutritional support for patients with liver disease to improve nutritional status, liver function, mental status, and overall survival[96,97]. Nutritional support also reduces the incidence of complications after LT[96,97]. However, a recent Cochrane review of 37 RCTs found no significant difference in mortality in patients with advanced liver disease receiving either enteral or parenteral nutritional support *vs* those receiving neither[95]. The investigators did, however, note improvement in serum bilirubin level, nitrogen balance, and hepatic encephalopathy; and reduced incidence of post-operative complications, particularly infections. The reviewed RCTs generally had methodological flaws, which could have caused overestimation of the observed effects[95]. The American Association for the Study of Liver Diseases (AASLD) and American College of Gastroenterology (ACG) recommend regular assessment of patients for nutritional, vitamin, and mineral deficiencies; appropriate supplementation for identified deficiencies; enteral nutritional therapy for severe ALD; and frequent interval feedings, emphasizing breakfast and a nighttime snack[32]. The diet should include 1.2-1.5 g of protein/kg per day and 35-40 kcal/kg per day to improve nitrogen balance[32]. These recommendations revise prior recommendations to reduce protein intake to prevent hepatic encephalopathy, in order to maintain positive protein balance and prevent muscle wasting in cirrhotics[98].

Therapies for alcoholic hepatitis

Although nutritional and supportive management are important, the mainstay of therapy for AH remains alcohol abstinence. Treatment varies according to severity. Patients who have DF < 32 have only 10% 28 d mortality without treatment[55]. Supportive management is adequate for such patients. However, patients who have DF ⩾ 32 have mortality between 30%-60% without treatment[48-54], and treatment may be lifesaving in this population.

Treatment options for severe AH include corticosteroids and pentoxifylline, which are well established, albeit controversial, therapies. Other treatment options, including infliximab, etanercept, antioxidants, and complemen-

Table 2 Meta-analyses of randomized controlled trials of corticosteroid therapy *vs* placebo for severe alcoholic hepatitis

Ref.	Inclusion criteria	Number of RCTs (total number of patients)	Endpoint parameters (primary endpoint listed on first line of each entry)	RR, HR or OR for primary endpoint	95%CI	Comments
Imperiale *et al*[102], 1990	RCTs of patients with acute AH receiving corticosteroids *vs* placebo	11 (562)	Mortality Hepatic encephalopathy	RR = 0.63	0.5-0.8	Positive study (P = 0.025)
Christensen *et al*[101], 1995	RCTs evaluating short term effect on survival of treatment with glucocorticoids *vs* placebo for AH	13 (659)	Mortality Age Serum bilirubin Ascites Male gender Hepatic encephalopathy	RR = 0.78	0.51-1.18	Negative study (P = 0.2)
Mathurin *et al*[55], 2002	RCTs during 1984-1992 of patients receiving glucocorticoids *vs* placebo	3 (215)	Survival Age Liver function tests DF Hepatic encephalopathy Gender Serum creatinine Ascites Leukocyte count	OR = 0.39	0.22-0.71	Positive study (P = 0.002) Used individual patient data analysis to increase statistical rigor for the meta-analysis
Rambaldi *et al*[100], 2008	RCTs of patients with severe, clinically overt AH diagnosed by clinical and biochemical criteria, treated with glucocorticoids *vs* placebo (or no intervention)	15 (721)	Mortality Liver-related mortality Symptoms and complications Liver function tests Liver histology Adverse events	RR = 0.83	0.63-1.11	Negative study (P = 0.21)
Mathurin *et al*[99], 2011	RCTs from 1984 to 2006 with specific data on DF ⩾ 32 or hepatic encephalopathy, of corticosteroids *vs* placebo, enteral nutrition or antioxidants	5 (418)	Survival DF Lille score Liver function tests	Complete responder: HR = 0.18	Complete responder: 0.05-0.71	Positive study Complete responders (P = 0.005)
			Serum creatinine Ascites	Partial responder: HR = 0.38	Partial responder: 0.17-0.87	Partial responders (P = 0.03)
			Hepatic encephalopathy Age	Null responder: HR = 0.81	Null responder: 0.45-1.45	Null responders (P = 0.46)
			Gender Leukocyte count			Used individual patient data analysis to increase statistical rigor for the meta-analysis

RCT: Randomized controlled trials; AH: Alcoholic hepatitis; DF: Discriminant function.

tary medicines have not been shown to improve clinical outcome[24].

Corticosteroids: Corticosteroids are the oldest and most investigated pharmacologic therapy for severe AH. Several RCTs have been performed with contradictory results[48-54]. Ramond *et al*[49] reported 12.5% mortality among patients receiving prednisolone *vs* 55% mortality among patients receiving placebo (P = 0.001). Contrariwise, a large study of 178 patients by Mendenhall *et al*[50] reported no statistically significant difference in mortality among patients treated with corticosteroids *vs* placebo. Both studies had limited statistical power because of small-to-moderate study size. Several investigators performed meta-analyses of the numerous RCTs to increase statistical power[55,99-102]. Three of these meta-analyses showed improvement in short-term survival in AH patients receiving corticosteroid treatment[55,99,102], whereas two meta-analyses showed no improvement[100,101] (Table 2). For example, a Cochrane meta-analysis incorporating 721 randomized patients demonstrated no statistically significant improvement in mortality among AH patients treated with corticosteroids; however, a subgroup analysis performed on low-bias risk trials revealed significantly reduced mortality in corticosteroid-treated patients with either DF ⩾ 32 or hepatic encephalopathy (RR = 0.33, 95%CI: 0.11-0.97)[100]. One affirmative meta-analysis performed individual patient data meta-analysis, considered the gold standard for meta-analysis with the least bias[103]; it showed that AH patients receiving corticosteroids had a 28-d mortality of only 20% *vs* 34% for controls (P = 0.0005)[99]. Furthermore, a prior meta-analysis demonstrated the importance of corticosteroid therapy in AH patients, showing that the number needed to treat (NNT) with corticosteroids

to prevent one death was only five[55]. Additionally, corticosteroids, specifically prednisolone, have been shown to significantly reduce mortality for at least 1 year[104].

The meta-analyses generally included only RCTs that analyzed patients with either DF ≥ 32 or hepatic encephalopathy, but none of the individual RCTs analyzed whether there was a maximum severity of AH (*e.g.*, maximum DF) beyond which patients no longer benefited from corticosteroid therapy. One study[99] analyzed this question and noted that patients respond rapidly (≤ 7 d) to corticosteroids with sustained response until the conclusion of treatment. They stratified patients according to Lille score as complete responders (score ≤ 0.16), partial responders (score = 0.16-0.56), and null responders (score ≥ 0.56). Survival benefit was limited to patients who were partial or complete responders; therefore, this study suggests modifying corticosteroid therapy according to therapeutic response[99].

Clinical application of corticosteroid therapy for AH is currently limited by insufficient data on its molecular therapeutic mechanisms. However, in a recent study of mice heavily exposed to alcohol for 10 d, administration of prednisolone, a corticosteroid, enhanced ethanol-induced liver injury and fibrosis compared to untreated controls[105]. This study further investigated potential mechanisms for the deleterious effects of prednisolone after hepatotoxic injury. In carbon tetrachloride-induced liver injury in mice models, prednisolone led to attenuation of macrophage and neutrophil functions that normally help clear apoptotic cells and resolve hepatic inflammation, and caused delayed hepatocyte regeneration by inhibiting expression of genes involved in hepatocyte proliferation and repair, such as pSTAT3[105]. These data may help modify and improve clinical management of corticosteroid therapy for AH.

Despite variable survival benefit among studies, no severe complications were reported in patients receiving corticosteroid therapy, including no significantly increased risk of infections. Corticosteroid therapy should not be precluded in patients who have preexistent infections, after initiation of appropriate antibiotic therapy[106]. Although the current data are somewhat contradictory, use of corticosteroids is generally recommended as first-line therapy for severe AH[26,32].

Pentoxifylline: Pentoxifylline, a nonspecific phosphodiesterase inhibitor, has anti-inflammatory properties, including inhibition of tumor necrosis factor (TNF)-α, that may retard hepatic inflammation and fibrosis[107]. It prevents development of hepatopulmonary syndrome and development of a hyperdynamic circulatory state in cirrhotic rats[108]. It decreases the risk of hepatorenal syndrome (HRS), and significantly improves renal function in patients with severe AH or cirrhosis[107,109-111]. In a study of 50 patients, it decreased DF by > 50%, *vs* a decrease of only 7.1% in patients receiving placebo (P = 0.001)[109].

Despite these positive effects, improved survival from pentoxifylline remains controversial. Four RCTs of pentoxifylline *vs* placebo reported contradictory findings (Table 3)[107,109,110,112]. Two trials showed no statistically significant improvement in short-term survival[107,112]; a third trial showed statistically significant improvement[110]; and a fourth trial showed a statistically insignificant trend towards improved short-term survival (20% mortality with pentoxifylline *vs* 40% with placebo) (P = 0.216)[109]. A Cochrane review, incorporating 5 RCTs, was inconclusive, and recommended further RCTs on this drug[113]. Another meta-analysis, incorporating 884 patients, supported previous findings that pentoxifylline decreases the risk of severe HRS, but failed to demonstrate significantly improved survival[111]. Further research with larger patient studies is necessary to determine whether pentoxifylline improves survival. Nevertheless, the AASLD, ACG, and the European Association for the Study of the Liver (EASL) recommend pentoxifylline as a second-line therapy in patients with severe AH who have contraindications to corticosteroid therapy[26,32].

Combination therapy: Corticosteroids or pentoxifylline are used in patients with severe AH, but the survival benefit for either drug remains controversial. Investigators combined both therapies to assess whether combination therapy is superior to corticosteroid monotherapy, especially for severe AH. However, two published RCTs found no significant difference in 6 mo mortality between combination therapy *vs* monotherapy[114,115]. The most recent RCT revealed 6-mo mortality of 30.1% in combination therapy *vs* 30.8% in monotherapy (P = 0.91)[115]. Although one RCT reported a significantly lower cumulative risk of HRS in the combination therapy group at 1 mo (P = 0.007)[115], the benefit was no longer significant at 6 mo[114,115]. Further studies are needed to explore combined treatment options to improve survival for severe AH.

Liver transplantation

LT is the only cure for end-stage ALD. Recent data from Organ Procurement and Transplantation Network (OPTN) and Scientific Registry of Transplant Recipients (SRTR) reveal 3563 alcoholic cirrhotics awaiting LT, constituting 23.2% of all patients awaiting LT[116]. Alcoholic cirrhosis is the second most common indication for LT, after cirrhosis from viral hepatitis[117]. Patients with alcoholic cirrhosis, however, have less likelihood of LT than other patients due to social factors. ALD is pejoratively thought as a "self-inflicted" disease, and donor organs are said to be better allocated to patients with other forms of end-stage liver disease (ESLD), which are not self-inflicted[118-120]. The critical, legitimate concern regarding LT for alcoholic cirrhosis is recidivism, with consequent disease recurrence in the allograft[117-121]. From 12%-46% of alcoholic cirrhotics resume alcohol consumption after LT[118-121]. The precise definition of recidivism var-

Table 3 Published randomized controlled trials of pentoxifylline *vs* placebo for severe alcoholic hepatitis *n* (%)

Ref.	*n*	Duration of treatment with pentoxifylline 400 mg PO *tid*	Mortality in placebo	Mortality in pentoxifylline	Relative risk or hazard ratio	95%CI	Comments
Akriviadis *et al*[110], 2000	101	28 d	24/52 (46)	12/49 (24)	RR = 0.59	0.35-0.97	Positive study (P = 0.037)
Fernández-Rodríguez *et al*[112], 2008	24	28 d	Not reported[1]	Not reported[1]	HR = 1.46	0.5-4.28	Negative study (P = 0.48)
Tyagi *et al*[107], 2011	61[2]	6 mo	2/31 (6)	1/30 (3)	Not reported	Not reported	Negative study[2] (P = 0.15)
Sidhu *et al*[109], 2012	50	28 d	10/25 (40)	5/25 (20)	RR = 0.5	0.19-1.25	Negative study (P = 0.216)

[1]Fernandez-Rodriguez *et al*[112] reported no statistically significant difference in short-term or long-term survival based on actuarial survival curve; [2]Tyagi *et al*[107] randomized 70 patients, but only 61 completed follow-up and were included in the analysis. The study did not show a significant difference in mortality, but showed a significant difference in the occurrence of hepatorenal syndrome. PO: Per overall survival; RR: Relative risk; HR: Hazard ratio.

ies among studies, and the rate of significant alcohol consumption post-transplant remains unclear[26]. Risk factors for recidivism include short duration of alcohol abstinence before LT, alcohol consumption just before LT, and patient denial of alcoholism[120,121]. To prevent recidivism, AASLD recommends LT candidates undergo assessment by an addictive behavior specialist, and delay of LT for ≥ 6 mo after commencing abstinence[70]. Six mo of abstinence may occasionally permit sufficient clinical improvement to render LT unnecessary[32]. EASL also supports this recommendation, and mandates a multidisciplinary approach, including psychological assessment, in addition to medical evaluation, to determine suitability for LT[26].

LT patients with alcoholic cirrhosis have at least comparable and perhaps even better survival than LT patients with other etiologies of ESLD[117,119,120]. For example, Burra *et al*[117] reported 1, 3, 5, and 10 years graft survival rates after LT in ALD patients to be 84%, 78%, 73%, and 58%, respectively; these rates are significantly higher than that for cirrhosis from viral hepatitis (P = 0.04) or cryptogenic cirrhosis (P = 0.05). Major causes of post-transplant mortality include infections, cardiovascular events and *de novo* malignancies[117], associated with immunosuppression[122,123]. *De novo* malignancies account for 23% of mortality in patients with alcoholic cirrhosis, compared to 11% of mortality in patients with cirrhosis from viral hepatitis without alcoholism or cryptogenic cirrhosis (P < 0.0001)[117]. The cumulative risk for *de novo* malignancies rises from 6% prior to LT to 55% 15 years after LT[26]. Therefore, regular screening for certain malignancies, including skin cancer, the most frequent post-transplantation cancer, is recommended[122].

NATURAL HISTORY AND COMPLICATIONS OF ALCOHOLIC CIRRHOSIS

Long-term management of complications of alcoholic cirrhosis is similar to that for other etiologies of cirrhosis (Table 4)[124-131]. Ascites is the most common complication of cirrhosis. Patients with new-onset ascites should undergo diagnostic paracentesis, to exclude other etiologies of ascites, such as cardiac disease, malignant ascites, or nephrotic syndrome; and to exclude spontaneous bacterial peritonitis (SBP). Ascitic fluid should be analyzed for cell count and differential, total protein, and serum-ascites albumin gradient (SAAG). A SAAG ≥ 1.1 g/dL supports the diagnosis of ascites secondary to portal hypertension; a SAAG < 1.1 g/dL supports other etiologies for ascites[132]. First-line treatment of ascites from cirrhosis includes alcohol cessation; dietary sodium restriction to ≤ 2 g/d; oral diuretics, usually spironolactone and furosemide; and discontinuation of non-steroidal anti-inflammatory drugs (NSAIDs)[129]. In patients who develop hyponatremia, reduction of diuretic dose or its temporary discontinuation may be necessary[127].

Patients with refractory ascites may warrant second-line treatment, including serial, large-volume, therapeutic paracenteses, TIPS, and addition of midodrine, especially in patients with systemic hypotension[129]. Midodrine, an α-adrenergic agonist, improves systemic hemodynamics by causing arterial vasoconstriction (reversing arterial vasodilation that contributes to development of ascites)[127]. β-blockers, angiotensin converting enzyme inhibitors, and angiotensin receptor blockers are no longer recommended for patients with ascites from cirrhosis, because of risks of life-threatening systemic hypotension[129]. Infusion of 6-8 g of albumin per liter of removed ascitic fluid is recommended during large-volume paracentesis (removal of > 5 liters of ascitic fluid)[129]. Peritoneovenous shunts used to be popular to treat refractory ascites, but are now restricted to patients with diuretic-resistant ascites who are poor candidates for serial paracenteses, transplantation, or TIPS, because of risks of disseminated intravascular coagulation and/or sepsis[129,133-135].

SBP, a complication of ascites, is diagnosed by presence of ≥ 250 polymorphonuclear cells/mm^3 in ascitic fluid. In the appropriate clinical setting, patients should receive empiric antibiotic therapy, preferably cefotaxime 2 g every 8 h, immediately after performing aerobic and anaerobic cultures of ascitic fluid[129]. Delaying antibiotic therapy to await culture results may cause life-threatening, overwhelming infection. Ascitic fluid culture is not

Table 4 Management of complications of cirrhosis, per professional society guidelines[1]

Complication	Screening/diagnosis	Treatment	Long-term management surveillance
Ascites	Diagnostic paracentesis for new-onset ascites: ascitic fluid analyzed for cell count and differential, total protein, and SAAG	Alcohol cessation; dietary sodium restriction; oral diuretics; discontinuation of NSAIDs	Refractory ascites: periodic large-volume therapeutic paracenteses; TIPS; midodrine; or peritoneovenous shunts
Spontaneous bacterial peritonitis	Diagnostic paracentesis: ≥ 250 polymorphonuclear cells/mm^3	Empiric antibiotic therapy with cefotaxime 2 g every 8 h, while awaiting culture results	Prophylaxis with norfloxacin or trimethoprim-sulfamethoxazole after one documented episode of SBP or if patient presents with variceal bleeding
Esophageal and gastric varices	Esophagogastroduodenoscopy	Treatment depends upon size of varices or risk of variceal bleeding: Prophylaxis with nadolol or propranolol for small varices at high risk of bleeding or for medium/large varices; EVL for medium/large varices at high risk of bleeding	No varices: EGD every 3 yr (earlier if hepatic decompensation occurs) Small varices: EGD every 2 yr Medium/large varices: EGD every 6-12 mo
Hepatic encephalopathy	Diagnosed by serum ammonia level and clinical findings of confusion, personality and mental status changes, and asterixis (exclude other causes of mental status changes)	Investigation and correction of precipitating factors; lactulose and/or rifaximin, supportive care	Secondary prophylaxis with lactulose and/or rifaximin indefinitely
Hepatorenal syndrome (type 1-rapidly progressive renal insufficiency; type 2-slowly progressive renal insufficiency)	Serum creatinine > 1.5 mg/dL, in the absence of other identifiable cause of renal failure (exclude other causes by urine chemistries, urine culture, and/or renal imaging)	Initial fluid challenge; albumin and terlipressin or albumin and combined octreotide plus midodrine; dialysis; LT definitive	Serial serum creatinine monitoring
Hepatocellular carcinoma (HCC)	Abdominal ultrasound every 6 mo; alpha fetoprotein determination every 6 mo no longer recommended, but optional	For HCC treatment[124]	Abdominal ultrasound every 6 mo
Hepatopulmonary syndrome	Screening by arterial blood gas; Confirmation by CEE	Symptomatic management with long-term oxygen therapy; LT definitive	
Portopulmonary hypertension	Screening by transthoracic Doppler echocardiography; Confirmation by right heart catheterization	Intravenous or inhaled prostacyclin; long-term oxygen therapy	

[1]American Association for the Study of Liver Diseases[124,128,129]; American College of Gastroenterology[125,126]; Department of Veterans Affairs Hepatitis C Resource Center Program and the National Hepatitis C Program[127]; European Association for the Study of the Liver[130]; and European Respiratory Society[131]. SAAG: Serum-ascites albumin gradient; NSAID: Non-steroidal anti-inflammatory drugs; TIPS: Transjugular intrahepatic portosystemic shunt; SBP: Spontaneous bacterial peritonitis; GI: Gastrointestinal; EGD: Esophagogastroduodenoscopy; EVL: Endoscopic variceal ligation; CEE: Transthoracic echocardiography with contrast enhancement.

necessary for diagnosis because up to 60% of patients have negative cultures[129]. Risk factors for persistent SBP include MELD score > 25, SAAG > 1.5, and positive ascitic fluid culture[136]. After one episode of SBP, patients should receive long-term prophylaxis with norfloxacin or trimethoprim/sulfamethoxazole[129]. Cirrhotic patients presenting with GI bleeding should also receive SBP antibiotic prophylaxis, with either intravenous ceftriaxone or oral norfloxacin, for 7 d[129].

Cirrhotic patients should undergo screening esophagogastroduodenoscopy (EGD) to diagnose or exclude esophageal and gastric varices. Classification of varices has been simplified to: small varices - minimally elevated veins above esophageal mucosal surface; medium varices - tortuous veins occupying < 1/3 of esophageal lumen; and large varices - tortuous veins occupying > 1/3 of esophageal lumen[128]. Patients with no varices undergo repeat EGD every 3 years, or sooner if hepatic decompensation occurs. Patients with small varices at increased risk of bleeding should receive primary prophylaxis with nonselective β-blockers, such as propranolol or nadolol, and should undergo EGD every 2 years. Patients at high risk of bleeding includes those with red wale markings at EGD or those with CTP stage B or C cirrhosis[128]. Patients with medium/large varices should also receive primary prophylaxis with nonselective β-blockers, but should undergo endoscopic variceal ligation when at high risk for variceal bleeding. This high-risk population should undergo surveillance EGD every 6-12 mo[127,128].

Hepatic encephalopathy is a potentially reversible neuropsychiatric disturbance resulting from hepatic insufficiency. It is characterized by confusion, personality and mental status changes, asterixis, and hyperammonemia. Hepatic encephalopathy is staged according to West-Haven criteria as: 0, normal; 1, mild; 2, lethargy; 3, somnolence-to-stupor; and 4, coma[126]. Precipitating factors, including GI bleeding, infections, electrolyte disturbances (especially hyponatremia), medications (primarily

narcotics and sedatives), constipation, and excessive nitrogenous dietary intake, should be assiduously investigated and corrected[126]. Acute pharmacologic management includes lactulose and/or rifaximin therapy[125,126]. Lactulose, a non-absorbable disaccharide cathartic, reduces nitrogenous load in gut, thereby reducing ammonia production[126]. Rifaximin, an antimicrobial agent with minimal systemic absorption, reduces ammonia-producing enteric bacteria. Rifaximin has been shown to reduce the risk of breakthrough hepatic encephalopathy during a 6 mo period of remission and to be superior to lactulose in treating hepatic encephalopathy[137]. Supportive management includes fall prevention, nursing care, prophylactic intubation in cases of severe hepatic encephalopathy, and adequate nutritional support[126]. After recovery, patients require secondary prophylaxis indefinitely with lactulose, rifaximin, or combination therapy to prevent recurrence.

HRS is characterized by serum creatinine > 1.5 mg/dL in a patient with ESLD, in the absence of other identifiable causes of acute or chronic renal failure[130]. It is a diagnosis of exclusion. It is classified into: type 1 HRS, characterized by rapidly progressive impairment in renal function (100% increase in baseline creatinine or creatinine level > 2.5 mg/dL, usually within 2 wk); and type 2 HRS, characterized by slowly progressive (> 2 wk) worsening renal function[127,130]. All cirrhotic patients with sudden increases in serum creatinine to > 1.5 mg/dL should have discontinuation of diuretics and receive a fluid challenge with 1.5 L intravenous normal saline[138]. Patients who are prerenal respond to fluid challenge, with decrease in creatinine levels and improved urine output, whereas patients with HRS are mostly unresponsive to fluid challenge[127,138]. Other causes of renal toxicity should be excluded, such as NSAIDs, hypotension, hypovolemia, obstructive uropathy, and sepsis. First-line therapy for HRS includes albumin and terlipressin, a vasopressin analogue that improves splanchnic circulation[130], or albumin and combined octreotide plus midodrine[127]. Patients not responding to these therapies may require dialysis and subsequent LT (usually with simultaneous renal transplant), the only definitive treatment for HRS. Pentoxifylline, as aforementioned, can decrease the incidence of HRS[107].

HCC occurs in 5%-15% of patients with alcoholic cirrhosis[23]. The precise incidence is, however, uncertain, as patients with alcoholic cirrhosis are often co-infected with HCV, which acts synergistically to potentiate the risk of HCC[124]. Nevertheless, screening for HCC is recommended in all patients with alcoholic cirrhosis. HCC surveillance is usually performed by abdominal ultrasound every 6 mo. Surveillance with serial serum alpha-fetoprotein determinations is no longer recommended because of insufficient specificity and sensitivity[124], but is still frequently performed. Diagnosis and treatment are similar in all patients with HCC, regardless of etiology of cirrhosis. The reader is referred to a comprehensive review on this subject[124].

Pulmonary vascular complications of chronic liver disease include hepatopulmonary syndrome (HPS) and portopulmonary hypertension (PPHTN). HPS is defined as an arterial oxygenation defect caused by intrapulmonary vascular dilatation in patients with liver disease, especially cirrhosis[139]. It occurs in approximately 20% of patients awaiting LT. Symptoms include dyspnea and platypnea, a characteristic finding of increased shortness of breath on rising from supine to upright position[131]. Screening, using arterial blood gas, is done in LT candidates and patients with liver disease who present with such symptoms. Transthoracic echocardiography with contrast enhancement (CEE) is the gold standard for diagnosis of HPS. CEE is commonly accomplished by hand agitation of 10 mL normal saline, resulting in microbubbles ($\leqslant$ 90 μm in diameter), which are injected into an upper extremity vein[131]. Detection of microbubbles within the left atrium is considered a positive CEE[131]. Diagnostic criteria include: (1) presence of liver disease; (2) alveolar-arterial oxygen tension difference (A-a gradient) $\geqslant$ 15 mmHg; and (3) positive CEE[131]. The only curative treatment for HPS is LT, but patients can be managed symptomatically with long-term oxygen therapy.

PPHTN is defined as pulmonary artery hypertension associated with portal hypertension, likely secondary to imbalance in vasoactive substances reaching the pulmonary circulation from portosystemic shunts or defective hepatic metabolism[140]. It occurs in approximately 5% of patients awaiting LT. Patients present with dyspnea, chest discomfort, or syncope. Transthoracic Doppler echocardiography is used for screening; PPHTN is suspected by finding of right ventricular systolic pressure > 40-50 mmHg. Diagnosis is confirmed by right heart catheterization, that reveals: (1) mean pulmonary artery pressure > 25 mmHg; (2) mean pulmonary artery occlusion pressure < 15 mmHg; and (3) pulmonary vascular resistance > 240 dyn-s/cm^{-5}[131]. Treatment may include pulmonary vasodilator therapy with intravenous and inhaled prostacyclin, as well as long-term oxygen therapy. LT is reserved for patients who fail to improve with these therapies.

FUTURE DIRECTIONS

Current research on ALD involves non-invasive diagnosis of ALD and novel treatment options. Non-invasive diagnostic liver tests, including FibroScan, have been studied, but the diagnostic accuracy of these tests have not been compared to the gold standard of liver biopsy, to define cut-off values for ALD[141]. The current prognostic scoring systems account for only 75%-85% of mortality for ALD or other causes of liver disease. Scoring systems incorporating more linearly independent variables may increase prognostic accuracy.

Currently, LT remains the only curative treatment. Further studies are needed for currently popular therapies, such as corticosteroids and pentoxifylline, to deter-

mine efficacy, especially to prove survival benefit. Combined medical therapies may be useful to achieve synergy in improving survival.

New treatment options that target pathways implicated in ALD pathogenesis, including oxidative stress, endotoxin production, cytokine production, and immune regulators[12] are being investigated. Recently investigated antioxidants include milk thistle (silymarin extracts) and *S*-adenosyl-*L*-methionine, both of which have not been proven beneficial[142,143]. However, most studies have been of low quality, and high quality RCTs regarding these relatively nontoxic, and possibly helpful treatments should be performed. Other TNF-α inhibitors, including etanercept and infliximab, have been studied, but only 3 RCTs with small study sizes have been published on these medications[144-146]. Reported adverse events and likely increased mortality limit their use, but further studies may be needed to confirm these findings.

Interleukin-22 (IL-22) is a potential therapy for ALD[14]. IL-22 ameliorates hepatic steatosis and liver injury in animal models after acute or chronic-binge ethanol feeding[147]. It may promote hepatocyte proliferation or hepatic regeneration and inhibit hepatic fibrosis in response to alcohol-induced liver injury[147]. IL-22 theoretically appears to be relatively safe because only hepatocytes, epithelial cells, and a few other cell types have IL-22 receptors. IL-22, however, promotes proliferation of preexisting hepatomas, even though it does not initiate hepatoma formation[148]. It is therefore likely contraindicated in patients with ALD complicated by hepatoma and may have limited use in patients with alcoholic cirrhosis.

Increased intestinal permeability to gut-derived microorganisms appears to increase morbidity and mortality in AH[149]. Several multi-institutional consortia are developing therapies for AH based on preventing or neutralizing these effects of increased intestinal permeability. For example, lipopolysaccharide (LPS) antibody may help neutralize injury from lipopolysaccharide from exposure to gut-derived microorganisms. One study will compare the effects of lipopolysaccharide (LPS) antibody in combination with corticosteroids *vs* corticosteroid monotherapy in patients with severe AH[149]. Other studies will examine the efficacy of probiotics *vs* placebo for moderately severe AH, or the effect of adding zinc, a mineral that improves gut barrier function, to other therapies for severe AH.

Another promising approach to AH therapy is targeting macrophage/Kupfer cell activation in AH which leads to increased IL-1 beta activation. A clinical trial is examining a combination of Anakinra, an interleukin 1 receptor antagonist, and traditional therapy *vs* traditional therapy alone for severe AH[150]. Another attractive approach is to inhibit caspases which are death induction molecules downstream to TNF-alpha activation during hepatotoxic injury. Emricasan, a pancaspase inhibitor, is proposed to be tested to block hepatocyte injury induced by TNF-beta, without blocking the beneficial hepatic effects of TNF-beta on liver regeneration and immune cell function[150]. Other novel potential therapies are in the process of development or undergoing preliminary clinical trials[14].

CONCLUSION

ALD is a prominent and preventable cause of morbidity and mortality. The cornerstone of therapy is abstinence, which improves overall survival. Psychological and pharmacologic therapies can support abstinence. Nutritional and supportive therapies are also important. Several therapies for AH, such as corticosteroids and pentoxifylline, are widely administered, but their survival benefit remains unproven. These drugs are generally well tolerated, without significant toxicity. Other potential therapies include TNF-α inhibitors other than pentoxifylline, antioxidants, and complementary medicine, none of which have demonstrable benefits for ALD and are not recommended as therapies. LT remains the only definitive treatment for alcoholic cirrhosis, and multidisciplinary management, including aggressive psychosocial therapy to prevent relapse should be instituted post-transplant. Patients with advanced ALD have complications that are similar to cirrhosis of other etiologies. Prophylaxis, surveillance, and aggressive treatment are important to prevent significant morbidity and mortality.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (10): Alcoholic liver disease

Pharmacotherapy of acute alcoholic hepatitis in clinical practice

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Abstract

Severe alcoholic hepatitis (AH) is an acute form of alcohol induced liver disease with a poor prognosis that is seen in the patients who consume large quantities of alcohol. The diagnosis of AH is based on the appropriate alcohol intake history and is supported with clinical and histological features, and several scoring systems. Glucocorticoids are the mainstay for treating severe AH with pentoxifylline used as an alternative to steroids in addition to total alcohol abstinence. Liver transplantation is a possible therapeutic option for severe AH. Among the anti-craving medications able to improve abstinence rate, baclofen seems to be effective and safe in the alcoholic patients affected by severe liver damage.

Key words: Severe alcoholic hepatitis; Maddrey's discriminant function; Glucocorticoids; Baclofen; Orthotopic liver transplantation; Alcoholic liver disease

Core tip: The therapy of severe alcoholic hepatitis (AH) is a problem in clinical practice due to the complex of the pathogenetic mechanisms involved. However, several treatment options are now available. The specific treatment of AH is directed to acute injury in order to block the progression of the fibrosis. Orthotopic liver transplantation is a possible therapeutic option for severe AH in the non-responder patients. Baclofen seems to be effective and safe anti-craving drug able to improve abstinence in patients with severe AH.

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INTRODUCTION

Alcohol use disorders (AUD) is a major cause of preventable morbidity and mortality worldwide[1]. Severe alcoholic hepatitis (AH) is a serious form of alcohol-related liver injury that is seen in the patients who consume large quantities of alcohol during a prolonged period of time[2]. The term "acute alcoholic hepatitis" was first used by Beckett *et al*[3] in 1961 and included a spectrum of the severity ranging from the asymptomatic mild abnormalities of liver chemistry tests to the fulminant liver failure and even death. However, the term "acute" represents a rapid worsening of an underlying chronic liver disease, and severe AH is defined as an acute-on-chronic

liver failure in many patients. It remains important for the clinicians to detect and treat this complex disease competently and satisfactorily.

The risk of the alcoholic liver disease (ALD) increases with the dose and duration of alcohol consumption[4]. However, the history of this condition is also influenced by many host factors, in particular obesity[5], female gender[6], viral co-infection (*i.e.,* chronic hepatitis C infection)[7], and iron overload[8], that are well known to increase the risk significantly[9].

The acute ingestion of alcohol can cause several metabolic alterations, including hypoglycemia, lactic acidosis, hypokalemia, hypomagnesemia, hypoalbuminemia, hypocalcemia, and hypophosphatemia on different body parts and tracts[10,11]. Acute alcohol intoxication-related cardiovascular effects include tachycardia, peripheral vasodilation and volume depletion; these features can contribute to the induction of hypothermia and hypotension[12]. Another possible cardiovascular effect is the "holiday heart syndrome", characterized by atrial or ventricular tachyarrhythmias and a new onset of atrial fibrillation after the acute alcohol ingestion[13]. The main life-threatening respiratory consequence of acute alcohol intoxication is respiratory depression[14]. Other respiratory effects include decreased airway sensitivity to foreign bodies, decreased ciliary clearance and aspiration and increased risk of bacterial infection with consequent bronchitis and pneumonia. Gastrointestinal effects include nausea, vomiting, diarrhea, abdominal pain due to gastritis, peptic ulcer, and pancreatitis[15]. Prolonged vomiting can lead to hyponatremia. Acute alcohol intoxication can cause a dysfunction of esophageal, gastric, and duodenal motility and an increase in duodenal type III propulsive waves in the ileum; the increased transit of the intestinal contents may contribute to diarrhea[16]. Excessive alcohol consumption is also a risk factor for developing colorectal adenomas or colorectal cancer[17].

The symptoms are usually related to the blood alcohol concentration (BAC). At the BAC higher than 300 mg/dL (65.1 mmol/L), there is an increased risk of respiratory depression and arrest. The death attributable to acute alcohol intoxication generally occurs at the BAC higher than 500 mg/dL (108.5 mmol/L), although the lethal dose of alcohol can vary. Specifically, death was observed at lower BACs in the "non-tolerant" subjects (300 mg/dL; 65.1 mmol/L) and the recovery was reported at higher levels (> 1200 mg/dL; 260.4 mmol/L)[18]. However, in the alcohol-dependent patients who develop a tolerance to alcohol as a result of the repeated exposure to ethanol, these effects may become reduced. This phenomenon seems to be related to the compensatory changes in excitatory *N*-methyl-*D*-aspartate and inhibitory gamma-amino-butyric acid (GABA)[19].

Acute alcohol intoxication can induce AH, usually in the subjects with chronic AUD and/or in the patients affected by alcoholic cirrhosis. Physical findings in the patients with AH include jaundice (the principal sign), hepatomegaly and spider angiomas. The symptoms may be non-specific which may involve fever, anorexia, weight loss, right upper quadrant pain, distension, or nausea and vomiting[20]. Alternatively, more severe symptoms can include encephalopathy and ascites.

PATHOGENESIS

The spectra of ALD are grouped into three histological stages: fatty liver, AH, and chronic hepatitis with fibrosis or cirrhosis. Fatty liver, the earliest response of the liver to alcohol abuse, is generally reversible with abstinence and is not believed to predispose to any chronic form of the liver disease if abstinence or moderation is maintained. AH develops in the patients with steatosis and is characterized by the presence of the inflammatory cells and hepatocellular injury with progressive fibrosis. In this case the reversibility is related to the degree of the liver injury. Finally, cirrhosis is irreversible and involves replacement of the normal hepatic parenchyma with extensive thick bands of fibrosis and regenerative nodules, which results in the clinical manifestations of portal hypertension and liver failure[21,22].

The toxic effect of alcohol on the liver is done by direct toxicity and an inflammatory cascade arising from portal venous translocation of Gram-negative bacteria due to increased small bowel permeability. The subsequent activation of Kupffer cell causes the release of reactive oxygen species and the production of various pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL) -7, IL-8, CXCL1, that lead to parenchymal neutrophil infiltration. Acetaldehyde, the alcohol metabolite, forms a variety of protein/DNA adducts that promote glutathione depletion, lipid peroxidation and mitochondrial damage[23].

DIAGNOSIS AND PROGNOSIS

AH generally occurs after decades of heavy AUD. Serum aminotransferase activities are typically five-to eight-fold elevated, the aspartate aminotransferase to alanine aminotransferase ratio is typically > 2, and serum bilirubin and alkaline phosphatise levels are generally elevated[3,18]. The differential diagnosis of suspected AH includes biliary obstruction, decompensated alcoholic cirrhosis, foamy fatty change, Zieve syndrome, drug-induced liver disease and acute viral hepatitis (*i.e.* hepatitis A or E). Serum bile acid concentrations are correlated with histology of AH in the patients with biopsy-proven disease. However, no consistent association between AH and cholestasis scores was observed[24].

The AH histopathological findings are characterised by the coexistence of macrovesicular steatosis, centrilobular ballooning of hepatocytes, infiltrate with polymorphonuclear leukocytes, Mallory bodies, megamitochondria and canalicular bile plugs[25]. The true reason of the incidence of AH is unclear. The published guidelines recommend histological confirmation of severe AH in the cases of diagnostic uncertainty[26]. Liver biopsy remains a useful tool in the diagnosis and

Table 1 Common scoring system used to predict prognosis in alcoholic hepatitis

Score name	Score formula				8- to 30-d mortality
Maddrey's discriminant function[31]	4.6 × [PT (s) - lab control PT (s)] + serum bilirubin (mg/dL)				Sensibility: 0.75 Specificity: 0.69
Model for end-stage liver disease[32]	3.78 × [Ln serum bilirubin (mg/dL)] + 11.2 × [Ln INR] + 9.57 × [Ln serum creatinine (mg/dL)] + 6.43				Sensibility: 0.69-0.75 Specificity: 0.68-0.75
Glasgow alcoholic hepatitis score[33]	Score given	1	2	3	Sensibility: 0.67 Specificity: 0.70
	Age	< 50	⩾ 50	-	
	WCC (10^9/L)	< 15	⩾ 15	-	
	Urea (mmol/L)	< 5	⩾ 5	-	
	PT ratio or INR	1.5	1.5-2.0	> 2.0	
	Bilirubin (μmol/L)	< 125	125-250	> 250	
ABIC score[34]	(age × 0.1) + [serum bilirubin (mg/dL) × 0.08] + [serum creatinine (mg/dL) × 0.3] + (INR × 0.8)				Sensibility: 0.92 Specificity: 0.32
Lille model score[35]	exp(-R)/[1 + exp(-R)][1]				Sensibility: 0.76 Specificity: 0.66

[1]*R* = 3.19-0.101 × (age in years) + 0.147 × (albumin day 0 in g/L) + 0.0165 × [bilirubin day 0 - bilirubin day 7 (mmol/L)] - 0.206 × (renal insufficiency) - 0.0065 × (bilirubin day 0 in mmol/L) - 0.0096 × (PT in seconds). PT: Prothrombin time; INR: International normalised ratio; WCC: White cell count.

management of severe AH, particularly when medical therapy is contemplated[27,28]. The prevalence of AH in the patients who undergo liver biopsy is of about 20% and it may be present in as many as 10%-35% of the hospitalized alcoholic patients[29,30]. Mild and moderate forms of AH frequently respond to alcoholic abstinence, whereas the prognosis of severe AH is poor; up to 40% die within 6 mo[31]. Especially in severe AH, even in the absence of cirrhosis, the portal system may come under the increased pressure because of liver scarring, resulting in portal hypertension and its complications.

The decision on how and when to treat the condition is pivotal and depends on the ability to establish the prognosis of the patients. Several scoring systems are available to assess the severity and prognosis of AH (Table 1). In particular, the modified Maddrey's discriminant function (MDF), the model for end-stage liver disease (MELD) score, the Glasgow alcoholic hepatitis score and the age-bilirubin-INR-creatinine (ABIC) score are utilized in the clinical practice[32-34].

The purpose of these scoring systems is to estimate the likelihood of short-term survival and to determine whether the patient should be treated with corticosteroids. The Lille score, instead, helps the physician to make the decision to stop corticosteroids after a week, or to continue for 28 d[35]. All scores use total bilirubin. The "weak point" of MDF is that requires the prothrombin time (PT) for the calculation. However, PT value can change between different laboratories[28,32]. This evidence has led to the development of new scoring systems. The MELD score, Glasgow, ABIC, and Lille score, all incorporating a measure of a kidney function, underscore the prognostic significance of an impaired kidney function in the patients with AH. In particular the MELD score includes the INR, which is standardized across laboratories, whereas the PT is not, and weighting of the INR and bilirubin level to reduce the influence of values at extremes[32].

MDF is the simplest and the most widely used score, validated by several groups as a reproducible criterion to identify the patients at a high risk of early mortality. Its score allows identification of those with non-life threatening AH (MDF < 32) who will recover with abstinence and who do not require specific treatment. Those with higher scores experience mortality of up to 50% in some studies and the recent clinical trials have addressed the management of the patients in this group. International guidelines report the use of MDF to estimate the likelihood of short-term survival as the primary endpoint. The MELD, the Glasgow, and the ABIC scores may be considered as alternative or additional tools to assess the disease severity.

PHARMACOTHERAPY

The optimal pharmacological treatment of severe AH is controversial and is one of the main challenges in the ALD. The development of the specific treatments has followed increasing understanding of the pathogenesis of this disease[36,37]. The key processes involve oxidative stress, inflammation and fibrosis. Secondary abnormalities include malnutrition and impaired hepatic regeneration. The specific treatment of the ALD is directed to acute injury in order to block the progression of the fibrosis. With a lifestyle modification, some studies support the treatment with glucocorticoids (GCs), pentoxifylline, anti-TNF-α, *S*-adenosylmethionine (SAMe) and antioxidants (Table 2).

Glucocorticoids

GCs are the first line treatment for severe AH. However, the efficacy of steroids has been debated for several decades and considered as the potential side effects that include anti-anabolism, muscular proteolysis, immunosuppression, increased susceptibility to the infections and increased risk of gastrointestinal bleeding. In addition, many patients with alcoholic diseases are predominantly obese, insulin resistant, or diabetic, and concomitant chronic hepatitis B or C is often present. In these settings, the clinical management with steroids are very

Table 2 Treatment considerations of severe alcoholic hepatitis

Treatment	Options	Comments
Corticosteroids	Prednisolone	If MDF ⩾ 32: 40 mg daily orally for 28 d followed by a 2/4-wk taper
Phosphodiesterase inhibitors	Pentoxyfilline	400 mg orally 3 times daily for 4 wk
Anti TNF-α	Infliximab	Infliximab 5 mg/kg *iv* at day 0 and prednisone 40 mg/d for 28 d (data not confirmed)
Nutrition	Eating, tube feeding	Diet rich in carbohydrate- and protein-derived calories; potassium replacement; vitamin supplementation
Antioxidant	Metadoxine	1500 mg/d orally for 3 mo
Antioxidant	*S*-adenosylmethionine	1200 mg/d orally in ambulatory patients
Alcohol abstinence	Rehab program	Reduce alcohol withdrawal symptoms, alcohol craving and intake, promote abstinence, evaluation for OLT program
	disulfiram, naltrexone, acamprosate, baclofen	

MDF: Maddrey's discriminant function; OLT: Orthotopic liver transplantation; TNF-α: Tumor necrosis factor-α.

difficult.

A meta-analysis at the beginning of the 90s, done by 11 randomized studies (10 of which were placebo controlled), suggested a beneficial role of GSs in the patients with severe AH with hepatic encephalopathy, but without active gastrointestinal bleeding by reducing short-term mortality[38]. A later analysis involving 12 controlled clinical trials, could not confirm these benefits, including the patients with encephalopathy[39]. In further support of this finding, the Cochrane review of 2008, including 15 randomized controlled trials with a total of 721 patients, concluded that GCs did not statistically reduce mortality compared with the placebo. A mortality benefit, however, was seen in a subgroup of the patients with MDF greater than 32 or with hepatic encephalopathy[40]. A successive meta-analysis was done using individual patient data from 5 randomised controlled trials. The patients were classified as complete responders (Lille score ⩽ 0.16), partial responders (Lille score between 0.16 and 0.56), and null responders (Lille score ⩾ 0.16). GCs improved 28-d survival in the patients' survival within the complete and partial responders, but not in the null responders[41].

Based on these data and on the clinical guidelines of the European Association for the Study of the Liver it is possible to make appropriate conclusions[42]. The GCs therapy of AH is limited by the concerns of heightened risks of sepsis and gastrointestinal hemorrhage. The patients with mild to moderate AH (MDF < 32), without hepatic encephalopathy and with the improvement in serum bilirubin or decline in the MDF score during the first week of hospitalization, should be monitored closely. Such patients will not likely require the benefit from the specific medical interventions and are expected to recover with nutritional support and abstinence from alcohol. The patients with severe disease (MDF ⩾ 32) with or without encephalopathy, who do not have contraindications to GCs, should be considered for a 4-wk treatment with prednisolone (40 mg/d), stopped or followed by a taper during 2-4 wk. Prednisolone is preferred to prednisone because the latter requires conversion to prednisolone in the liver, a process that may be impaired in AH. A new treatment is needed for the poor responders. Early liver transplantation may be considered after a careful selection process of these patients.

Pentoxifylline

Pentoxifylline is a phosphodiesterase inhibitor that blocks transcription of TNF-α to decrease serum levels of the gene product. It may be an acceptable therapeutic option in the patients with severe AH. When compared to the placebo, the patients with severe AH (MDF score ⩾ 32) treated with pentoxifylline presented a higher 6-mo survival. This was related to a marked decreased development of a hepatorenal syndrome[43]. Three randomized clinical trials compared pentoxifylline in combination with GCs and GCs monotherapy in severe AH[44-46]. However, these studies reported that the combination of GCs and pentoxifylline presented no additional survival advantage compared with GCs alone in a 6-mo survival. On the basis of these data, the guidelines by American Association for the Study of Liver Disease recommend pentoxifylline (400 mg orally 3 times daily for 4 wk) in the patients with severe AH (MDF score ⩾ 32), especially if there are contraindications to the GCs treatment[26,47]. The European Association for the Study of the Liver Guidelines recommend using pentoxifylline if sepsis precludes the use of GCs[42].

Anti TNF-α

TNF-α is a key cytokine that reproduces a number of features of alcoholic hepatitis. It is known to be increased in proportion to the severity of this disease and its low levels increase the liver regeneration[48]. The anti TNF-α therapies were considered as the most attractive strategies to treat severe AH. In a pilot study, 20 patients with biopsy-proved severe AH (MDF between 32 and 55) were randomized to either 5 mg/kg *iv* of infliximab at day zero plus 40 mg/d of prednisone or prednisone alone. In severe AH, infliximab was well tolerated and associated with a significant improvement of MDF score at day 28[49]. The effectiveness of anti-TNF-α was not confirmed in two randomized controlled trials testing multiple doses of infliximab (10 mg/kg in weeks 0, 2, 4, associated with prednisolone 40 mg/d for 28 d) or etanercept (25 mg *sc* 6 times over 3 wk), for association with a higher probability of severe infections and deaths[50,51]. In summary, the role of anti TNF-α agents

is limited in the confines of the approved randomized clinical trials.

Metadoxine

One specific drug that is useful in the treatment of severe AH is metadoxine (pyridoxol *L*-2-pyrrolidone-5-carboxylate). Pyrrolidone carboxylate is involved in the amino acid metabolism through the glutathione pathway[52]. It facilitates de novo ATP synthesis and prevents ATP decrease in the brain and liver of rats acutely intoxicated with ethanol. Pyridoxine increases the metabolic degradation rate of ethanol, thereby reducing the damage to the cell functions caused by acetaldehyde, the first metabolite in the ethanol elimination process. It can also prevent glutathione depletion, lipid peroxidation damage, collagen deposition and TNF-α secretions induced by alcohol and acetaldehyde in hepatocytes and hepatic stellate cells[53]. A double-blind controlled trial included 136 alcoholic patients diagnosed with fatty liver who were randomly assigned to metadoxine (1500 mg/d) or placebo for 3 mo. A significant improvement in the liver function tests was reported in both groups at the end of the study. However, the improvement was observed more rapidly in those randomized to metadoxine[54]. More recently our group, in a retrospective study of 94 alcohol dependent patients, who received metadoxine for alcohol intoxication with a dose ranged between 500-2000 mg/d and a period of 2-42 d, registered a significant improvement in the transaminase levels, accompanied by the decrease in drinks per week and craving level[53]. Further studies are needed for better understanding of the potential role of metadoxine to treat the ALD, and, in particular, the severe forms of AH, and to define the exact dosage.

S-adenosylmethionine and other antioxidant

SAMe is a principal methyl donor for methyltransferase reactions that regulates gene expression and facilitates the generation of the antioxidant glutathione from homocysteine. The protective effects on the liver in the course of alcoholic injury are also mediated by the maintenance of mitochondrial function and down-regulation of TNF-α. Several data established the association of an abnormal hepatic methionine metabolism with the development of the ALD[55]. SAMe was studied in the patients with liver cirrhosis. In a multicenter randomized, double-blind trial, 123 patients were treated with SAMe (1200 mg/d, orally) or placebo for 2 years. Mortality was not reduced overall, but after the exclusion of a small number of very advanced cases with Childs C cirrhosis, a significant reduction in mortality was found. This study has yet to be replicated, but SAMe is used sporadically on the basis of these encouraging results and its safe profile[56]. Randomized, blinded, placebo-controlled studies assessed the effectiveness of Milk Thistle in chronic ALD. The results of these trials might be conflicting and confounded because of heterogeneity of the degree of the disease severity and alcoholic intake or abstinence[57]. Pre-clinical studies showed that silymarin, the active complex of this plant, play a role to protect against the acute liver injury caused by ethanol administration[58]. Considering its safety profile, it could be developed as an effective therapeutic agent for acute AH by its anti-oxidative stress and anti-inflammatory features. For ambulatory patients, the antioxidant therapies may be considered in a motivated patient with a specific nutritional support.

OTHER TREATMENTS

Nutrition

Alcoholic patients present a profound catabolic state with malnutrition, secondary to anorexia and poor diet, which can promote bacterial infections[59]. In fact, large volumes of alcohol suppress the appetite. Many admit that drinking is the main source of their calories in the form of alcohol. However, nutritional support is recommended in the patients with AH[60]. It improves the liver function and the obtained results from histological analyses might increase the survival rates based on the results of short-term follow-up studies. Nutrition should be provided orally or *via* a nasojejunal tube if nausea, vomiting, or encephalopathy are present. The patients with AH also require multivitamin, folic acid and thiamine supplementations. The formula of the enteral diet was a low-fat diet in which medium-chain triglycerides and oleic acid were accounted for most of its lipid content and rich in carbohydrate and protein-derived calories[61]. It was also suggested that combined treatment with enteral nutrition and GCs could improve the outcome of the patients with severe AH[62].

The maintenance of *ev* fluids should be avoided. These patients are often profoundly potassium depleted due to the lack of an intake of potassium-containing foods and hyperaldosteronism due to their liver disease. The replacement of potassium may be required daily until the serum potassium level is normal without supplementation. If the patients with AH exhibit signs of fluid retention, but the blood urea nitrogen and creatinine are normal, spironolactone may be given, which increases urinary excretion of sodium, water and serum potassium[63]. Oral furosemide may, then, be added, once the serum potassium normalizes without further need for potassium supplementation. If azotemia occurs, diuretics should be discontinued and the patients should be evaluated for a hepatorenal syndrome. There may be a component of malabsorption of vitamin K due to jaundice in addition to poor synthesis of coagulation components by the diseased liver. Three daily doses of vitamin K (10 mg) intravenously or subcutaneously usually decrease the international normalized ratio (INR). Oral dosing of vitamin K is not effective because of the poor absorption in the setting of deep jaundice[37]. Finally, considering the potential risk of Wernicke's encephalopathy, the supplementation with B-complex vitamins is needed[64].

Baclofen

Total alcohol abstinence represents the cornerstone in the treatment strategy for the patients affected by severe AH. This point in the clinical practice is often problematic, especially when these patients present a psychiatric diagnosis of AUD. Medical recommendations and/or brief interventions may not be sufficient to achieve and maintain the abstinence when a diagnosis of dependence is present. Therefore, the need to add pharmacological approaches has been emphasized in the last decades. As a consequence, pharmacotherapy of AUD is undergoing a period of the scientific development. Several drugs that can reduce alcohol craving, and consequently, can increase abstinence and prevent alcohol relapse have been evaluated[65]. In particular, disulfiram, naltrexone, and acamprosate have been approved for AUD. However, these medications might worsen liver disease[66].

Baclofen is a $GABA_B$ receptor antagonist that represents a new alcohol pharmacotherapy. Several preclinical and clinical studies demonstrated that baclofen could represent an effective drug to treat the AUD patients[67,68]. In particular, this drug was shown to reduce alcohol withdrawal symptoms, as well as to reduce alcohol craving and intake and to promote alcohol abstinence[69]. Notably, baclofen showed a safe profile when administered to the alcoholics, including those with liver cirrhosis. In a randomized, double-blind, controlled study, we evaluated the efficacy of baclofen for the maintenance of alcohol abstinence in 148 alcohol-dependent patients with liver cirrhosis. The subjects were randomized to either oral baclofen (10 mg 3 times a day) or placebo for 12 wk. Of 42 patients treated with baclofen, 71% achieved and maintained abstinence compared with 29% of 42 patients assigned to the placebo group[70]. The appropriate dosing of baclofen is still being debated. The secondary analysis with baclofen in the patients without underlying liver disease, have shown a dose-effect relationship of the drug on the reduction of daily alcohol intake and on the number of drinks per drinking day[69]. In this regard, it should be taken into account that the safety of the drug in the alcoholic patients with alcoholic hepatitis has also been reported recently by Avanesyan[71].

Liver transplantation

Orthotopic liver transplantation (OLT) is a possible therapeutic option for severe AH in the non-responder patients. The OLT is the last treatment option for the patients with end-stage liver disease who have a greater than 10% risk of dying[42]. The patients with severe AH who do not respond to GCs or pentoxifylline have a mortality of 50% to 75% within 6 mo[72]. Several Centers have proposed that this is a rescue option for the patients with severe AH who do not respond to medical therapies. However, the risk of recidivism of AUD still represents the major ethical concern about the usefulness of the OLT in alcoholic patients. The outcomes and in particular the survival rate, are better than other causes of the end-stage liver disease, especially if recidivism does not occur[73]. Even when the recidivism is present, the risks of developing the ALD and graft loss are unpredictable, due to the modifications in the susceptibility to alcohol damage secondary to the OLT. A period of 6-mo in total alcohol abstinence before the OLT is proposed as a strategy to reduce the risk of recidivism. However, this strategy is not evidence based and it is not accepted worldwide[65]. Recently, a prospective multicenter study showed that the early OLT clearly improved the 6-mo survival in the patients with a first episode of severe AH not responding to medical treatment[74]. In our opinion, the 6-mo rule should not be considered as a predictor of a recidivism risk, but as a recovery period. In particular, with all its limitations, the 6-mo rule should be used to test the possible improvement of liver function, which could avoid the OLT. When liver function does not allow for a 6-mo waiting time, such as in the patients with severe AH or with the advanced ALD, the pre-OLT abstinence time should be shortened, at least in the patients strictly followed by an alcohol addiction unit (AAU)[74,75]. The management of the alcoholic patients with the end-stage ALD listed for the OLT by an AAU within a liver transplant Centre could represent a useful strategy to reduce the risk of alcohol recidivism both before and after the transplantation.

FUTURE PERSPECTIVES

ALD remains one of the major medical problems in the individuals with AUD. The therapy of ALD and, in particular, of severe AH is a problem in a clinical practice due to the complex of the pathogenetic mechanisms. However, several options are now available and the use of several system scores to define the severity of the disease can guide the physician to make a treatment strategy in the function of the short-term survival. Recently, several basic and pre-clinical studies have started to define the cellular mechanisms involved in the liver disease progression and injury severity in a better way, including the high rates of apoptosis, lipid peroxidation, generation of free radicals and depletion of antioxidant capacity of the liver[76,77]. However, the results in animal models do not reproduce all the pathological changes found in humans with severe forms of the disease. In addition, translational research using human samples have identified novel potential therapeutic targets, but the prevailing pathogenetic pathways involved in the ALD are not defined and the innovations in the treatment approach are far to coming.

To the contrary, the most important goal of the therapy and the prevention in the ALD is abstinence from alcohol. In this regard, several data confirm the role of baclofen to reduce alcohol craving, particularly in the patients with the severe ALD. This drug is very interesting because it has a low level metabolism by the liver and appears to have few side effects, and presents the ability to maintain significantly a higher number of the patients

in abstinence which is beneficial in the treatment of the liver disease progression and/or to consider for the admission in an OLT program. To date, baclofen represents the only anti-craving medication formally tested in a randomized clinical trial in the alcoholic patients affected by liver cirrhosis, although additional confirmatory studies are warranted.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (10): Alcoholic liver disease

Chronic liver inflammation: Clinical implications beyond alcoholic liver disease

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Abstract

Chronic alcohol exposure can lead to alcoholic liver disease, including hepatitis, cirrhosis and hepatocellular carcinoma, and chronic inflammation can simultaneously cause systemic medical illness. Recent evidence suggests that alcoholic liver disease is a predictor for liver-related diseases, cardiovascular disease, immunologic disease, and bone disease. Chronic inflammation in alcoholic liver disease is mediated by a direct inflammatory cascade from the alcohol detoxification process and an indirect inflammatory cascade in response to gut microflora-derived lipopolysaccharides (LPS). The pathophysiology of alcoholic liver disease and its related systemic illness is characterized by oxidative stress, activation of the immune cascade, and gut-liver interactions. Integrative therapeutic strategies for alcoholic liver disease include abstaining from alcohol consumption; general anti-inflammatories such as glucocorticoid, pentoxifylline, and tumour necrosis factor-α antagonist; antioxidants such as N- acetylcysteine; gut microflora and LPS modulators such as rifaximin and/or probiotics. This review focuses on the impact of chronic liver inflammation on systemic health problems and several potential therapeutic targets.

Key words: Alcoholic liver disease; Oxidative stress; Cardiovascular disease; Immunologic disease; Bone disease

Core tip: Beyond the natural course in the liver, alcoholic liver disease can be implicated in many health problems that affect the quality of life and disease progression. Evidence suggests that alcoholic liver disease is a predictor for liver-related diseases, cardiovascular disease, immunologic disease, and bone disease. Chronic inflammation in alcoholic liver disease and related systemic illness is mediated by a direct response to alcohol and an indirect inflammatory response. Alcoholic liver disease should be considered from the perspective of chronic inflammation. Accordingly, integrative therapeutic strategies including anti-inflammatory targeting are needed for alcohol-induced liver inflammation management and prevention of systemic medical problems.

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INTRODUCTION

Chronic alcohol consumption is a major risk factor for chronic liver disease worldwide. Cardinal features of alcoholic liver disease include simple fatty liver, alcoholic hepatitis, fibrosis or, more seriously, cirrhosis and hepa-

tocellular carcinoma. Alcohol has been recognized as a true hepatotoxin, an agent able to cause liver damage, for many years[1]. Although abstaining from alcohol is the primary recommendation for managing alcoholic liver disease, the chronic features of alcoholic liver disease and its progression can affect a patient's attitude toward consumption. Alcohol is an important cause of hepatocellular carcinoma in Korea in addition to the hepatitis B virus and the hepatitis C virus[2]. Additionally, up to 48% of cirrhosis-related deaths have been associated with alcohol in the United States[3].

Recent evidence has determined that inflammation is closely linked with development of alcoholic liver disease[3-7]. Acute inflammation as a defense against noxious stimuli is very important for homeostasis in the body, whereas chronic exposure to an agent that induces inflammation may cause a dysregulated or unresolved inflammatory response, which causes chronic inflammation. Finally, various inflammatory components can influence systemic medical conditions. The major sources of chronic low-grade inflammation in alcoholic liver disease are categorized as follows: a direct inflammatory cascade from the alcohol detoxification process and an indirect inflammatory cascade in response to gut microflora-derived lipopolysaccharides (LPS).

The liver plays a key role in detoxifying alcohol and its related toxic products and is also responsible for immunologic effects. However, chronic alcohol consumption can lead to alcoholic liver disease and simultaneous systemic medical illness because of chronic inflammation. Beyond the natural course in the liver, alcoholic liver disease can be implicated in many health problems that affect the quality of life and disease progression. Therefore, alcoholic liver disease should be considered from the perspective of chronic inflammation. This review focuses on the impact of chronic liver inflammation on systemic health problems and several potential therapeutic targets.

CARDIOVASCULAR DISEASE

Emerging evidence suggests that alcoholic liver disease predicts not only liver-related diseases, but also atherosclerotic cardiovascular disease (CVD). Recent data suggest that some pathogenic mechanisms are involved in atherosclerotic CVD. In patients with alcoholic liver disease, alcohol, acetaldehyde, and excessive free fatty acids (FFA) in hepatocytes generate an excess of reactive oxygen species (ROS). This formation leads to lipid peroxidation, cytokine production, and hepatic inflammation[8], which contribute to a higher oxidative-inflammatory response. Thus, alcoholic liver disease may actively involve chronic low-grade inflammation in the arterial wall[9]. Moreover, pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-8 (IL-8), and interleukin-17 (IL-17) are produced by Kupffer cells in the liver in response to LPS, which, in turn, play a key role in inducing acute phase reactants in the liver, such as C-reactive protein (CRP), ferritin, and amyloid A[10,11]. These inflammatory cascades can also synergistically or interactively contribute to arterial inflammation. Indeed, several epidemiologic studies have shown that various inflammatory markers, such as TNF-α and CRP, are elevated in patients with alcoholic liver disease[12-14]. Furthermore, chronic low-grade inflammation plays a crucial role in regulating arterial wall tone by affecting the release of nitric oxide (NO) and endothelin-1[15,16]. These cascades may cause endothelial dysfunction and alter arterial elastic properties, leading to arterial stiffness. In addition, when a hepatic cell is damaged, hepatic stellate cells also secrete angiotensin Ⅱ[17], a major pro-atherogenic and vasoconstrictive peptide that acts on the arterial wall. The overproduction of hematostatic factors such as plasminogen activator inhibitor-1 (PAI-1) may have a direct atherogenic effect on blood vessels[18] in patients with alcoholic liver disease. Lastly, chronic alcohol consumption has a tendency for increased plasma homocysteine levels, albeit the results are inconsistent according to amount and types of alcoholic beverage consumed, or underlying diseases[19]. However, hyperhomocysteinemia induced by chronic alcohol consumption may be one of the important risk factors for CVD[20,21].

IMMUNOLOGIC DISEASE

Recent research has shown that alcoholic liver disease may alter immune regulation, which can lead to immunodeficiency and autoimmunity[22]. Additionally, individuals with chronic alcohol consumption are more susceptible to bacterial pneumonia and septicemia[23-25]. There is also an increased incidence of pulmonary tuberculosis or human immunodeficiency virus (HIV) in patients with alcoholic liver disease[26-28]. In addition, less common infectious diseases such as meningitis, diphtheria, lung abscess, or cellulitis are more prevalent in alcoholic liver disease[27].

Alcoholic hepatitis and alcoholic liver cirrhosis have been associated with autoimmune properties[22]. Liver function in patients with alcoholic hepatitis can decrease for several weeks (at least two weeks) after abstinence from alcohol, and resuming drinking after recovering from alcoholic hepatitis can lead to more severe alcoholic hepatitis. In this regard, autoantibodies against the liver may be an important cause of the liver damage and scarring in alcoholic liver disease.

Other autoimmune diseases or allergic reactions are also seen in alcoholic liver disease. Immunoglobulin A (IgA) has been found in skin and kidney deposits as well as the liver in many patients with alcoholic liver disease[29]. Also, alcohol consumption contributes to immunoglobulin E (IgE)-mediated reactions in susceptible humans, such as individuals with food allergies or asthma[30]. Chronic alcohol use has been found to increase total IgE level[30-32]. Therefore, understanding altered immunity and cytokines in alcoholic liver disease can be

important for assessing potential immunologic risk and could provide insight into therapeutic targets.

BONE DISEASE

Hepatic osteodystrophy is abnormal bone metabolism that has been identified in association with chronic liver disease, including such conditions as osteopenia, osteoporosis, and osteomalacia. The prevalence of osteopenia in patients with alcoholic liver disease is between 34% and 48%, and the prevalence of osteoporosis is between 11% and 36%[33-35]. However, osteomalacia has rarely been confirmed in patients with chronic liver disease and low vitamin D level[36,37].

Bone is a dynamic tissue that is remodeled through constant bone resorption and formation. Bone turnover accounts for up to 15% of the annual renovation of total bone mass[38]. Decreased bone density, commonly seen in hepatic osteodystrophy, results from decreased bone formation or increased bone resorption. Bone mineral density, measured by dual energy X-ray absorptiometry, is reported with a *Z* score and *T* score; the former is used to compare the patient's bone mineral density with an age-matched mean value, and the latter is used to compare the bone mineral density with that of healthy young individuals. Osteopenia is identified when a *T* score ranges from -1.0 to -2.5, and osteoporosis is defined by a *T* score < 2.5. Osteomalacia is characterized by abnormal bone matrix mineralization, which can be confirmed by bone biopsy. These metabolic bone diseases are very common and can be important complications in patients with alcoholic liver disease. Although the mechanism of metabolic bone diseases remains complex and multifactorial, osteoclastogenic inflammatory cytokines, such as interleukin 1 (IL-1) and TNF-α, have been known to have a key role in the pathogenesis of bone metabolic disease that is related to alcoholic liver disease[39]. Early assessment and therapeutic intervention in patients with hepatic osteodystrophy can be important for minimizing future fracture risk and maintaining the quality of life.

PATHOPHYSIOLOGY

Oxidative stress

It is known that alcohol increases ROS, which are chemically reactive molecules that can damage various cellular components such as proteins, lipids, or deoxyribonucleic acid (DNA)[40]. Moreover, acetaldehyde, an intermediate alcohol metabolite, is a highly reactive compound and is highly toxic to hepatocytes, promotes glutathione depletion, lipid peroxidation, and mitochondrial damage[41-42]. Evidence suggests that oxidative stress can contribute to the development of alcoholic liver disease and has been associated with various major diseases including cardiovascular diseases, type 2 diabetes, neurodegenerative disease, and carcinogenesis[43-46].

Although multiple mechanisms are involved in alcohol-related ROS production, cytochrome P450 E21 and the mitochondrial electron transport chain are important targets[40,47,48]. Moreover, alcohol-derived ROS may directly trigger the systemic inflammatory response[49]. ROS could activate nuclear factor kappa B (NF-κB), which leads to production of inflammatory cytokines such as TNF-α. Alcohol-derived ROS may play a role in initiating a vicious cycle *via* the liver cell damage mechanism with additional inflammatory cytokines and ROS production[50]. Therefore, alcohol-derived ROS may be important for understanding systemic inflammation accompanied with alcoholic liver disease.

Activation of immunity

As described above, individuals with chronic alcohol consumption are more susceptible to immunodeficiency and autoimmunity. Understanding altered innate and acquired immunity in alcoholic liver disease may be important for assessing the potential risk of opportunistic infections and allergic diseases such as food allergies and bronchial asthma.

Alcohol consumption causes gut microflora dysbiosis and bacterial over-growth and ultimately increases gut permeability and the translocation of LPS from the gut to the liver. In Kupffer cells, gut microflora derived-LPS interacts with toll-like receptor 4 (TLR4), and pro-inflammatory cytokines and chemokines such as TNF-α, IL-8, IL-17 are produced, leading to the production of ROS and alcohol-induced liver damage[51,52]. Interestingly, activation of TLR4 also induces Kupffer cells to produce hepatoprotective cytokines such as IL-6 which reduces hepatocyte necrosis-associated inflammation, albeit having proinflammatory roles, and anti-inflammatory cytokines such as interleukin-10 (IL-10)[53]. However, long-term alcohol consumption may generate lipid peroxidation products such as malondialdehyde (MDA) as a result of ROS cascades, which can modify many proteins linked to the adaptive immune response[54,55]. Patients with alcoholic liver disease have increased levels of circulating antibodies against lipid peroxidation products and increased numbers of T and B cells in the liver, which contribute to adaptive immunity activation in alcoholic liver disease[54,55].

Gut-liver interaction

Optimal functioning of the gut-liver axis depends on healthy gut integrity and mucosal microflora and a healthy liver; however, chronic alcohol exposure impairs both gut and liver health. These changes affect each other and ultimately contribute to the increased blood levels of LPS, or endotoxemia, in patients with alcoholic liver disease. Major inducers of chronic low-grade inflammation in alcoholic liver disease are broadly summarized as a direct inflammatory injury from alcohol and its metabolites or an indirect inflammatory injury in response to LPS. The microflora-derived LPS enters systemic circulation in two different ways, either *via* a portal vein or through gastrointestinal lymphatic vessels[56,57]. Most LPS

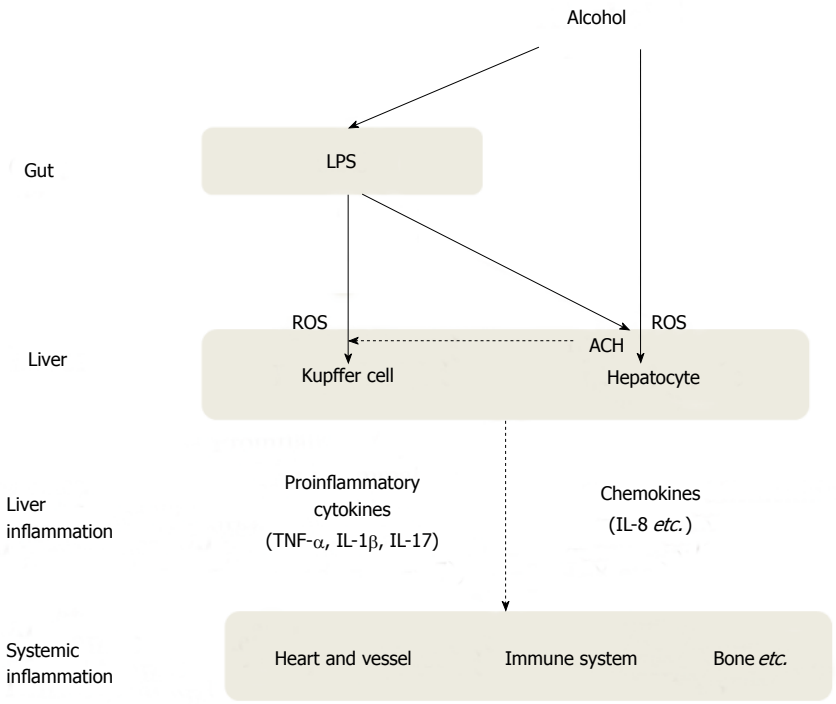

Figure 1 Schematic of liver inflammation. LPS: Lipopolysaccharides; ROS: Reactive oxygen species; ACH: Acetaldehyde; TNF-α: Tumor necrosis factor-α; IL-1β: Interleukin-1β; IL-17: Interleukin-17; IL-8: Interleukin-8.

in the lymphatic system move through mesenteric lymph nodes and eventually enter the systemic circulation at the thoracic duct opening, whereas most LPS in the portal vein can be detoxified and excreted.

Alcohol can alter gut integrity and permeability in both direct and indirect manners. Alcohol and its metabolites such as acetaldehyde can directly alter both gut permeability and microflora content and composition. Alcohol and acetaldehyde can weaken the intestinal epithelial barrier, such as tight junctions between intestinal enterocytes. Moreover, increased gut permeability in alcoholic liver disease may be aggravated by increased expression of inducible nitric oxide synthase (iNOS) and NF-κB, which, in turn, enhance the translocation of LPS between tight junctions of adjacent enterocytes[58,59]. This increased gut permeability is also called leaky gut syndrome (LGS). Patients with alcoholic hepatitis commonly show elevated LPS levels in plasma, implicating a crucial role of LPS-induced inflammation in the pathogenesis of alcoholic liver disease[60]. Thus, alcohol facilitates the translocation of endotoxin from the intestinal lumen to the portal vein, thereby aggravating the risk of liver injury.

Individuals with chronic alcohol use are more susceptible to small intestinal bacterial overgrowth and dysbiosis compared to counterpart non-alcoholics or abstainers[61,62]. Excessive alcohol ingestion facilitates the overgrowth of Gram-negative bacteria, contributing to increased endotoxin levels[60]. In addition, micronutrient deficiency, such as zinc, is common in alcoholic liver disease, which adversely affects the integrity of the intestinal epithelium[63]. More recently, evidence suggests that increased gut permeability may be an important factor in the pathogenesis of alcoholic liver disease.

As intestinal permeability increases, endotoxin and other bacterial toxins increase the sensitivity of Kupffer cells to LPS stimulation in the liver, where increased proinflammatory cytokines lead to neutrophil activation, increased sinusoidal permeability, generation of ROS, and mitochondrial damage in the liver[64]. These cascades may cause systemic low-grade inflammation in addition to liver inflammation (Figure 1).

THERAPEUTIC TARGETS

General anti-inflammatories

The first-line therapy for alcoholic liver disease is alcohol abstinence with nutritional support[3,7,65]. However, therapeutic lifestyle changes are not easy in clinical practice and may not be sufficient for some patients. Corticosteroids, pentoxifylline, and TNF-α antagonist have been identified as therapeutic agents for severe alcoholic hepatitis. Among them, corticosteroids and pentoxifylline are currently recommended[7]. Glucocorticoids could reduce immune activation by blocking cytotoxic and inflammatory signal pathways, and pentoxifyllin plays an anti-inflammatory role as a non-selective phosphodiesterase inhibitor and TNF-α suppressor[66]. Although TNF-α has been regarded as a predictor for the severity of alcoholic hepatitis and TNF-α antagonist reduces liver damage in alcohol-fed animals, clinical trials with TNF-α antibody have not shown consistent results[67-69].

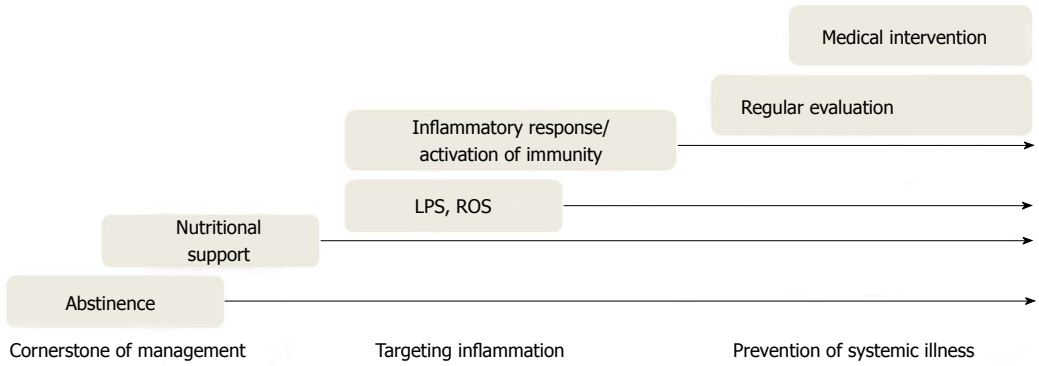

Figure 2 Alcohol-induced liver inflammation management and prevention of systemic medical problems. LPS: Lipopolysaccharides; ROS: Reactive oxygen species.

TNF-α antibody or corticosteroids may induce a condition that causes patients to be susceptible to infections because of immune suppression[7,69]. Pentoxifylline may be considered in patients with severe alcoholic hepatitis who cannot use corticosteroids[3].

Antioxidants

Antioxidants such as *N*-acetylcysteine have been reported to reduce inflammatory markers and liver fat accumulation in alcohol-fed animals[49]. *S*-adenosylmethionine could increase cellular antioxidant glutathione in patients with alcoholic liver disease[70]. Betaine, precursor to *S*-adenosylmethionine, has also been reported to attenuate alcoholic liver disease[71]. In clinical trials, *S*-adenosylmethionine has shown improved survival in patients with less advanced liver cirrhosis[72] but has not been consistently effective in treating alcoholic liver disease[73]. Antioxidants including phytochemicals such as resveratrol and carotenoids are successful for treating alcohol-fed animals, but lack convincing benefits in human patients[74-76]. Oxidative stress may be more pronounced in early stages of alcoholic liver disease, which is found in most animal models, but plays a minor role in later stages of alcoholic liver disease. Actually, administration of antioxidants cocktail has shown inferior survival rates compared to corticosteroid administration in patients with severe alcoholic hepatitis[77].

Gut microflora and LPS pathway

The gut-liver interaction has been identified as an important interaction for liver health and prevention of systemic inflammation. In this regard, the modulation of gut microflora and LPS pathway could be used to treat alcoholic liver disease[3,78,79] (Figure 2). For the former, probiotics and bioactive extracts may provide therapeutic benefit in patients with alcoholic liver disease[79,80]. In addition, non-absorbable antibiotics such as rifaximin and/or probiotics can modify the gut microflora and help reduce the risk of hepatic encephalopathy[81]. For the latter, TLR4 antagonists that modify the LPS pathway have been proposed as therapeutic materials for chronic liver disease[82].

Other therapeutic considerations

Several surrogate agents for treating alcoholic liver disease are being investigated. Global suppression of inflammatory responses could lead to undesirable side effects such as immune suppression. Therefore, specific anti-inflammatory targeting may be more promising. Recent studies have shown that IL-22 has hepatoprotective properties including antioxidant, anti-steatotic, and anti-microbial effects[83]. Moreover, the IL-22 receptor exists only on epithelial cells such as hepatocytes, and side effects that target this receptor may be minimal. Also, IL-8 and IL-17 have been related to neutrophil infiltration, TNF-α augmentation, and autoimmunity[7,84]. Therefore, based on the cytokine and immune cell profiles, specific intervention may merit serious consideration to reduce the inflammatory response with minimal side effects.

CONCLUSION

Chronic inflammation in alcoholic liver disease and related systemic illness is mediated by a direct response to alcohol and an indirect inflammatory response to gut microflora-derived LPS, leading to a stronger oxidative-inflammatory response. In addition to alcohol abstinence, integrative therapeutic strategies to reduce inflammatory cascades may be needed to treat and prevent alcoholic liver disease.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (13): Gastrointestinal endoscopy

Endoscopic ultrasound guided fine needle tissue acquisition: Where we stand in 2013?

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Abstract

Since its introduction, endoscopic ultrasound (EUS) guided fine needle aspiration and fine needle biopsy have become an indispensable tool for the diagnosis of lesions within the gastrointestinal tract and surrounding organs. It has proved to be an effective diagnostic method with high accuracy and low complication rates. Several factors can influence the accuracy and the diagnostic yield of this procedure including experience of the endosonographer, availability of onsite cytopathology services, the method of cytopathology preparation, the location and physical characteristics of the lesion, sampling techniques and the type and size of the needle used. In this review we will outline the recent studies evaluating EUS-guided tissue acquisition and will provide practical recommendations to maximize tissue yield.

Key words: Endoscopic ultrasound; Fine needle aspiration; Endoscopic ultrasound guided sampling techniques; Cytological diagnosis; Core biopsy device; Gastrointestinal endoscopy

Core tip: The impact of the type and size of needles used for endoscopic ultrasound guided-guided tissue acquisition have been the center of recent studies aiming at maximizing tissue yield. In addition to needles, several other variables impact the final outcome of tissue acquisition including the location and characteristics of the lesion, the fine needle aspiration technique, and the availability of on-site cytopathology services. In this review we outline the results of these studies and summarize the recent advances in this field.

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INTRODUCTION

Endoscopic ultrasound guided fine needle aspiration (EUS-FNA) was initially described in 1992[1] and soon became the procedure of choice to obtain diagnostic samples from lesions within the GI tract and regional orga[2-4]. EUS-FNA is highly accurate, sensitive and specific with estimates reaching 80%, 90% and 100% respectively for cytological diagnoses[5-8]. However, the diagnostic accuracy of EUS-FNA can be influenced by several factors including the experience of the endosonographer, the availability of onsite cytopathology review, the method of cytopathology preparation, the location and physical characteristics of the lesion, and type and size of the needle[9-13]. Currently, three needle sizes are commercially available including 19-G, 22-G and 25-G[2]. To choose a particular needle size, one should consider the location and the type of lesion targeted for sampling, in addition to the type of sample desired; whether a cytological or histological sample is necessary

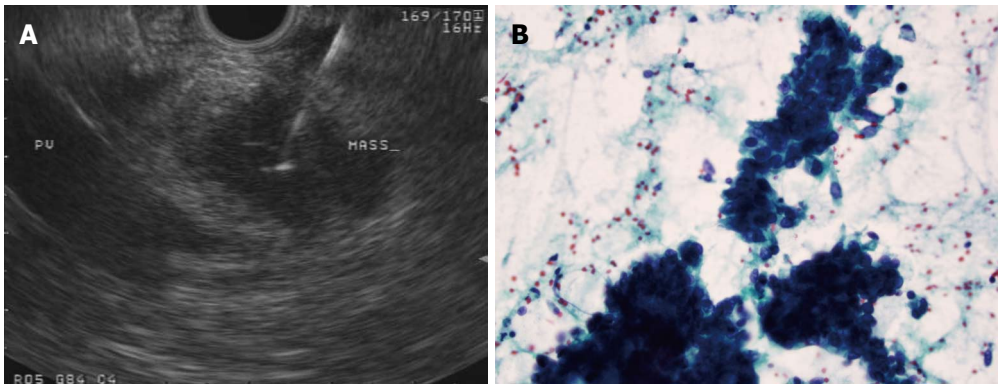

Figure 1 A 25-G needle is used to aspirate a 2 cm pancreatic head mass that does not appear to encase the portal vein (A), adenocarcinoma confirmed on wet smears obtained from the first pass in the case above (B). Papanicolaou stain, × 40.

to establish the diagnosis. Currently, the 22-G needles are probably the most widely used; however, a recent trend toward increased utilization of the smaller 25-G needle has been observed in many centers, particularly in scenarios where a transduodenal sampling is considered. Theoretically, larger needles can provide larger size tissue samples; however, technical difficulties are more frequently encountered with larger needles. This is largely related to the stiffness of the needle, leading to sampling failures of lesions located in areas that require significant angulation of the echoendoscope. Larger needles also carry higher risk of complications[14] and could increase the "bloodiness" of sample, which can make the diagnosis by cytology more challenging.

The type of lesion also impacts the choice of needle to be used. For example, stromal tumors and lymphomas can be difficult to diagnose by cytology alone, and sometimes require samples with preserved tissue architecture to make a diagnosis[15,16]. Obtaining an adequate histological sample is theoretically difficult with smaller needles. To overcome this, a Trucut biopsy needle (TCB: Cook Medical, Bloomington, IN, United States) was developed using larger size 19 G needles[17]. However, this needle was limited by difficulties encountered with larger needles such as stiffness, reduced maneuverability, and failure of the spring-loading mechanism, and therefore failed to establish itself when transduodenal approach for sampling was required[18]. More recently, a new generation of core biopsy devices of various sizes (ProCore, Cook Medical, Winston-Salem, NC, United States) in addition to another flexible 19-G needle made of nitinol (Expect 19-gauge Flex, Boston Scientific, Natick, MA, United States) were introduced to obtain histological samples including those from lesions that require transduodenal approach with promising results[19].

In addition to needle size, sampling technique can influence the quality of a specimen. Variations in sampling techniques utilized by endosonographers include the use of suction versus no suction and fanning technique to obtain specimens. Reinserting the stylet versus air flushing are techniques employed to express the sample prior to cytopathology exam.

In the following sections we will focus on recent literature comparing various needle types and sizes and their impact on quality of specimens in relation to the location and type of lesion. We will also review the different sampling techniques used by endosonographers and how such techniques may affect the quality of samples.

SAMPLING METHODS AND TECHNIQUES

Variations in technique of EUS-guided FNA have been recently assessed to identify the sampling method with the highest yield. Common examples to such methods include the use of suction, adopting the fanning technique, use of stylet, and expressing samples using air flushing or by reinserting the stylet.

Use of suction

The traditional FNA technique that relies on suction utilization was recently questioned. Lee *et al*[20] compared the quality and diagnostic yield of samples obtained with and without suction in 81 patients with pancreatic masses. In this study, each patient had specimens taken with and without applying suction. The number of diagnostic samples, cellularity, and accuracy were found to be higher in the suction group. In another trial with similar results[21], 52 patients with solid mass lesions were randomized to FNA with either suction or no suction. Sensitivity and negative predictive values were higher in the suction group compared to the non-suction group ($P = 0.05$).

Practically speaking, the decision to use suction should depend on the nature of the targeted lesion. In highly vascular lesions such as lymph nodes, a non-suction technique may result in a better quality and less bloody sample, particularly for the on-site cytopathologist to be able to render a preliminary diagnosis. On the other hand, applying suction when aspirating a fibrotic malignant lesion of the pancreas (Figure 1) or in the setting of chronic pancreatitis may provide a superior sample quality[22-24]. We recommend applying suction during the first pass and then tailoring the use of suction and the amount of based on feedback from the cytopathologist. It is always recom-

mended that aspiration should be applied in cystic pancreatic lesions to obtain sufficient fluid for cytology and tumor markers.

Use of stylet and fanning technique

It's widely assumed that using a stylet while going through the gut wall during the initial puncture helps prevent clogging of the needle's lumen by tissue from the wall, and potentially reduces the contamination of lesional tissue with GI wall components. Therefore, it remains a common practice to re-insert the stylet before every pass. Data comparing the adequacy of EUS-FNA with and without stylet remain limited. Wani *et al*[25] retrospectively compared EUS-FNA specimens obtained using the stylet to those obtained without using a stylet in terms of cellularity, contamination, adequacy, amount of blood and the diagnostic yield. No difference between the two techniques was found in relation to the variables studied. The authors' recommendation was against the use of stylet. The use of stylet is considered to be labor intensive and time consuming (particularly with 25-G needles), which could prolong procedure time.

When puncturing a lesion, endosonographers should attempt to sample multiple areas within the same lesion during every pass, a technique referred to as fanning. During this technique, the needle track is slightly altered during every from movement by modulating the up and down dial of the echoendoscope or by using elevator. Bang *et al*[26] compared this technique to the standard one in sampling 54 solid pancreatic masses, and found fanning to be superior by establishing diagnosis in fewer numbers of passes, and resulted in higher first pass diagnostic rate of (85.7% *vs* 57.7 %, $P = 0.02$).

Number of passes

To date, no definite number of passes to achieve the highest diagnostic yield has been established yet for various lesions. Nevertheless, increasing the number of passes has been associated with higher diagnostic yield[27]. Most studies have shown that 5-7 passes would be adequate in solid lesions[27,28]. In general, fewer passes are required when sampling highly vascular lesions such as lymph nodes compared to solid masses like pancreatic tumors[27,29]. For example, seven passes have resulted in a sensitivity and specificity of 83% and 100% respectively in solid pancreatic masses, while, in the case of lymph nodes, five passes provided a sensitivity and specificity of 77% and 100% respectively[30]. For cystic lesions, it is recommended that the lesion be completely aspirated until it collapses *via* a single puncture.

On-site cytopathology evaluation

The availability of rapid on-site cytopathology evaluation can improve the sampling process by reducing the number of passes needed and the frequency of inconclusive diagnoses. A feedback from an on-site cytopathologist can dictate whether additional passes are needed to procure a better quality specimen[31]. To evaluate the effect of an on-site cytopathology examination on sample quality and the need to repeat the procedure, Collins *et al*[32] compared cytological outcomes from pancreatic mass FNAs done in the presence of an on-site cytopathologist with those without. The presence of an immediate on-site cytopathology exam resulted in a significant impact on the diagnostic yield; where only 2.9% of the procedures needed to be repeated compared to 5.8% when an on-site cytopathologist was unavailable. Additionally, definitive diagnosis in the repeated procedures was achieved more frequently in procedures where an onsite cytopathology evaluation was present (67% *vs* 27%). Several other studies supported the presence of on-site cytopathology evaluation and showed improved adequacy of samples[33-35]. In comparison, fewer studies showed that the presence of an on-site cytopathology failed to translate into significant improvement to EUS-FNA outcomes[36,37].

Method of sample expression

The traditional method of expressing FNA samples is *via* air flushing the needle. Recently, this was compared to the method of reinserting the stylet in a study by Lee *et al*[20] Samples expressed by the two techniques were compared in terms of quality, cellularity and bloodiness. Bloodiness was less in the air-flushed group compared to the reinserting-the-stylet group ($P = 0.02$), but quality of samples and cellularity were similar in both groups. Similar results were reported by Rastogi *et al*[38] in a randomized controlled trial as well as by Sahai *et al*[39].

Reinserting the stylet remains a common practice despite being time consuming and could potentially be associated with increased risk of needle stick injury[2,9,38,39]. Based on the results of the above studies, stylet reinsertion could be reserved to conditions when the sample is dry or clotted and cannot be expressed by air flushing[20,33], which is not infrequent with 25-G needles.

Key points: Application of suction during sampling can increase the cellularity and the diagnostic yield particularly in solid fibrotic lesions. In highly vascular lesions a non-suction technique can reduce the bloodiness of the sample. Fanning technique can reduce the number of passes required to reach a diagnosis. Expression techniques have minimal impact on the sample's quality; however, air flushing seems to be less labor intensive. Use of stylet during the initial puncture or to express the sample has not been associated with improved specimen quality.

Needle size: FNA needles are commercially available in 19, 22 and 25-G sizes. Among all the variables that could impact the diagnostic outcome of EUS-FNA, needle size remains probably the most exhaustively evaluated. The most commonly used needle is the 22-G, although recently the 25-G needles have gained popularity in many centers due to their ease of use and recent data showing diagnostic equivalence compared to 22-G nee-

dles. In the following section we will discuss in details the choice of a particular needle size as it relates to the location of the lesion, type of the specimen and overall the quality of sample.

Location of the lesion

The location of the lesion can direct the sampling approach, and in some instances the size of the needle to be used. Approximately 65% of pancreatic cancers are found in the head or uncinate process[40]. Such tumors are best visualized and sampled through the duodenum, whereas lesions in the pancreatic body and tail are best evaluated through the stomach. In the duodenum, angulation of the echoendoscope tip is often required to maintain apposition with the mass. This position creates more resistance and makes the use of stiffer, larger 19-G needles challenging.

Itoi *et al*[41] objectively evaluated the resistance of 19-G Tru-cut, traditional 19-G, 22-G and 25-G aspiration needles during insertion and advancement under variable conditions of the echoendoscope (straight and angulated endoscope position, endoscope tip angulation, and while using of the elevator). In this trial, lower resistance was encountered with the 22-G and 25-G needles under almost every position compared to the conventional 19-G needle and 19-G Tru-cut needle. Additionally, the maneuverability of the scope itself was found to be reduced when using the 19-G needles.

To minimize resistance during transduodenal sampling, it is recommended to maintain the scope in a short position whenever possible. While the scope might be less stable at this position, it facilitates needle advancement out of the scope and penetration of the lesion. To improve stability of the scope, air suction or an inflated balloon could help bring the duodenal wall closer to the probe and further stabilize the tip of the scope during FNA.

Type of specimen

EUS-FNA remains the standard procedure for sampling pancreatic masses, in addition to other lesions like subepithelial tumors and enlarged lymph nodes. In most cases, cytology alone is adequate to reach a diagnosis. However, EUS-FNA has certain limitations. First, on-site cytopathology is not available in many centers. Second, certain conditions such as lymphoma, mesenchymal tumors, and well-differentiated adenocarcinomas can be difficult to diagnose by cytology alone[28,42], and a core biopsy (Trucut biopsy; TCB) with well-preserved tissue architecture may be essential for the diagnosis[43-45]. Third, the negative predictive value of EUS-FNA is relatively low, and therefore does not exclude malignancy in all negative specimens. In the last decade, a spring-loaded 19-TCB needle (Quick-core; Cook Medical, Winston-Salem, NC, United States) has been developed to overcome the limitations of FNA and procure larger quantities of tissue for histologic analysis[43]. Larghi *et al*[46] evaluated this needle in 23 patients with pancreatic masses. The overall success rate was 74% (17/23), however the success rate was significantly lower in transduodenal approach compared with transgastric approach. A larger study by Thomas *et al*[47] included 113 patients and showed a diagnostic accuracy of EUS-TCB of 68%. In this study there was no significant difference in the diagnostic yield for lesions procured by either transduodenal or transgastric approach. Based on a decade of experience, the use of this device has been mainly recommended in certain conditions where prior studies have shown better diagnostic compared to FNA yield such as lymphoma[48] and autoimmune pancreatitis[49]. The routine use of such device as an adjunct to FNA sampling was limited by the difficulty in operating it due to its stiffness and mechanical failures associated with its spring-loading mechanism.

To overcome some of the limitations associated with obtaining TCBs, a new series of fine needle biopsy needles has been developed (Procore, Cook Endoscopy, Winston Salem, NC) with reverse bevel technology to improve tissue acquisition. Iglesias-Garcia *et al*[44] evaluated the 19 G Procore needle of this series in a study that included 114 lesions. Adequate histological samples were obtained in 89% of lesions with overall diagnostic accuracy of over 85%. Transduodenal biopsy was successful in 33 of 35 cases (94%). However, technical difficulties were encountered when the needle was used transduodenally and in many cases the needle had to be advanced out of the scope in the stomach before reaching the duodenum. A prospective trial is currently underway to compare the performance and diagnostic yield of this and the older TCB device (Quick-core).

In another study, Bang *et al*[45] compared FNA using standard 22-G aspiration needle 22-G Procore needle for sampling solid pancreatic masses in 56 patients (Table 1). The trial showed no significant difference between the two devices in procurement of the core tissue (100% *vs* 83.3%, $P = 0.26$) or the presence of diagnostic histologic specimens (66.7% *vs* 80%, $P = 0.66$). No difficulty in performing transgastric or transduodenal biopsies was reported in this study using the ProCore needle. In another trial by Larghi *et al*[50], adequate histological samples were obtained in 54 out of 61 patients (88.5%) using the Procore needle.

The ability of standard FNA needles to provide adequate histological samples has been evaluated as well. In one trial, Rong *et al*[51] found that histologic adequacy of the standard 22-G needle was superior to the 25-G one in sampling pancreatic masses (70.4% *vs* 61.1%, $P = 0.33$) and submucosal tumors (74.1% *vs* 55.6%, $P = 0.18$). However, diagnostic accuracy did not differ between the two needles (80.0% *vs* 78.9%) when both needles were used on the same patient.

In evaluating the ability of the 19-G needle to obtain histological specimens, Yasuda *et al*[52] reported 98% accuracy of EUS-FNB using the conventional 19-G needle in diagnosing patients with unknown lymphadenopathy. In patients with lymphoma, the needle provided sufficient tissue to classify their lymphomas in

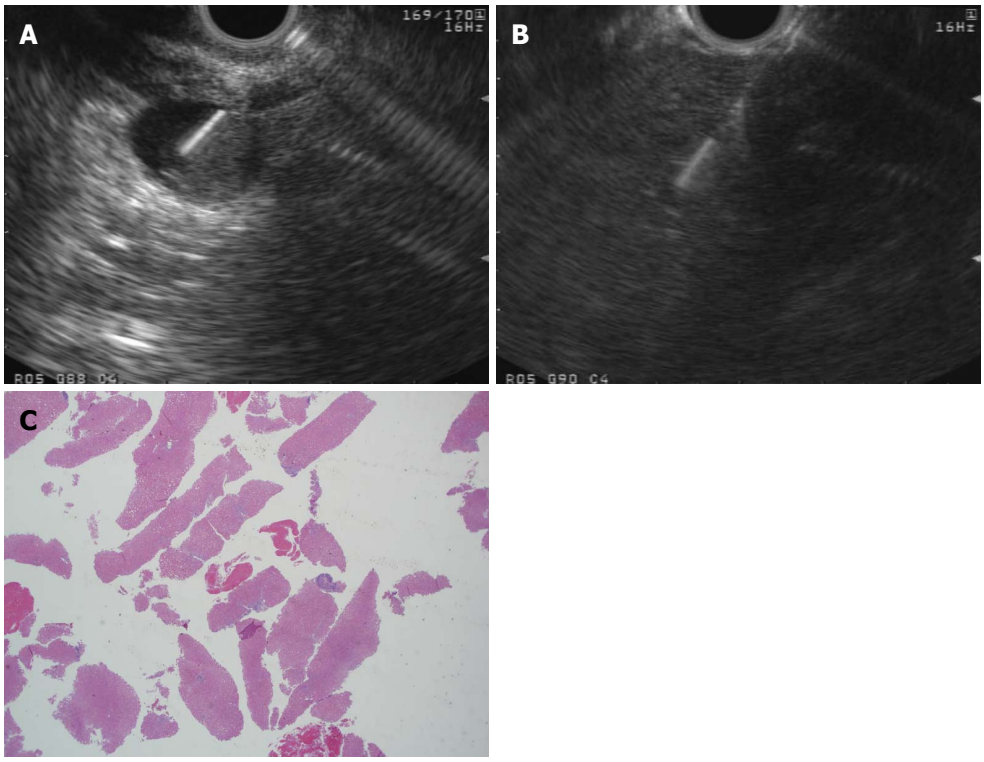

Figure 2 Flexible 19-G needle. A: 2 cm rectal subepithelial lesion was found to originate from the muscularis propria on endoscopic ultrasound guided and is sampled using a flexible 19-G needle in this figure; B: A core liver biopsy was obtained using a flexible 19-G needle is a patient with elevated transaminases; C: Histopathological assessment of the core biopsy obtained in the case above confirmed steatohepatitis without significant fibrosis. Adequate histopathology sample was obtained and stained positively for CD-117, confirming gastrointestinal stromal tumor; H and E stain, × 2.

Table 1 Rate of histologically adequate specimens procured using the 3 common size fine needles in recent literature

Ref.	Needle size	Number of patients	Histological adequacy	Location of biopsy
Bang *et al*[45]	22-G FNA	28	66.7%	Pancreas
	22-G FNB	28		
Yasuda *et al*[52]	19-G	104	98.0%	Lymph nodes
Rong *et al*[51]	22-G	54	70.4%	Pancreas
	25-G	54	61.1%	Pancreas
	22-G	27	74.1%	Submucosal tumors
	25-G	27	55.6%	Submucosal tumors
Larghi *et al*[53]	19-G	120	97.5%	Various
Varadarajulu *et al*[19]	19-G[1]	38	94.7%	Subepithelial masses Pancreatic (head and uncinate lesions)

[1]A flexible Nitinol based needle was used in all procedures. FNA: Fine needle aspiration; FNB: Fine needle biopsy.

accordance with the World Health Organization classifications in 88% of them. In another trial, adequate histological samples from solid masses were obtained in 97.5% of patients using the 19-G needle[53]. However, this study excluded patients with pancreatic head and uncinate masses that required transduodenal approach for sampling.

The stiffness of 19-G needles has been recently reduced in a new needle made of nitinol (Expect 19-gauge Flex, Boston Scientific, Natick, MA, United States). Nitinol is an alloy made of Nickel and Titanium, used in the construction of biliary endoprosthesis. The properties of this needle include resistance to deformation and high elasticity, which facilitate tissue sampling when the tip of the echoendoscope is in an angulated position. Two recent studies evaluated the clinical performance of this flexible 19-G needle (Figure 2)[19,54]. The first study by Varadarajulu *et al*[19] included 38 patients in whom 32 had pancreatic head or uncinate lesions. There were no technical failures reported in this trial, and histological samples were satisfactory in 94.7% of patients. In another multi-center trial published in abstract form, the needle was used in a variety of applications, with similar high technical success and histological adequacy rates[54].

Key points: Histological specimens are necessary for the diagnosis and appropriate classification of certain

Table 2 Quality of samples using different needle sizes as reported by recent studies

Ref.	Needle size	Number of patients	Location of lesion	Result
Lee *et al*[55]	22-G and 25-G	12	Pancreas and peripancreatic	No difference between the two needles in terms of cellularity (P = 0.84)
Siddiqui *et al*[56]	22-G and 25-G	131 (22-G = 64 patients) (25-G = 67 patients)	Pancreas	No significant difference in diagnostic yield (P = 0.18)
Fabbri *et al*[57]	22-G and 25-G	50	Pancreas	No significant difference in diagnostic accuracy 94% *vs* 86%
Imazu *et al*[58]	22-G and 25-G	43	Pancreas, lymph nodes, submucosal tumors	Similar overall diagnostic yield 22 > 25 in submucosal lesions (80% *vs* 60%) 25-G > 22-G in pancreatic lesions (91.5% *vs* 75%)
Camellini *et al*[59]	22-G and 25-G	127	Pancreatic, lymph nodes and subepithelial tumors	No significant difference in sample adequacy overall (77.8% *vs* 78.1%) Pancreatic lesions: 25-G > 22-G (87.8% *vs* 76.7%) Subepithelial lesions: 22-G > 25-G (55.5% *vs* 20%) Lymph nodes: 22-G > 25-G (100% *vs* 60%)
Sakamoto *et al*[18]	19-G, 22-G and 25-G	24	Pancreas	19-G and 22-G > 25-G in adequacy of samples for histological diagnosis 25-G had better diagnostic accuracy in pancreatic head and uncinate lesions
Song *et al*[61]	19-G and 22-G	117	Pancreatic and peripancreatic lesions	Sample quality and cellular material: 19-G > 22-G (P = 0.03)

conditions such as lymphoma, stromal cell tumors and well-differentiated cancers. New generation of fine needle core biopsy devices of various sizes facilitate transduodenal sampling and have been associated with high technical success rates and adequate histological sampling.

Quality of sample

Sample quality depends on several variables, including experience of the endosonographer, the availability of onsite cytopathology review, the method of cytopathology preparation, the location and physical characteristics of the lesion, and size of the needle[9-13]. Among all these factors, needle size continues to receive most attention as an independent factor that could impact the diagnostic yield of EUS-FNA. Comparing the quality of samples and diagnostic yield of the different needle sizes have been the focus of recent studies, most of which compared 22-G with 25-G needles[55-60] with fewer studies including 19-G needles[56,57] (Table 2).

Lee *et al*[55] (12 patients), Siddiqui *et al*[56] (131 patients) and Fabbri *et al*[57] (50 patients) compared the quality of samples and the diagnostic yield of the 22-G and 25-G needles in pancreatic lesions[55-57] and peri-pancreatic lesions[55]. The studies by Lee *et al*[55] and Fabbri *et al*[57] provided a comparison of the two needles in the same lesion. In all three studies, there was no significant difference in diagnostic yield between the two needles.

On the other hand, Imazu *et al*[58] found that the quantity of specimens obtained by the 22-G needle was overall higher than the 25-G needle, which resulted in a higher diagnostic yield in patients with submucosal tumors, where histological sample is essential for diagnosis. However, in patents with pancreatic lesions, the diagnostic yield of the 25-G needle was higher. This was believed to be the result of the tissue characteristics of solid pancreatic lesions which are typically hard in consistency and a smaller needle may provide an easier puncture and lead to better sample quality and less technical failures.

In a large prospective randomized study, Camellini *et al*[59] compared 22 with 25-G needles in 127 solid lesions with salvage crossover for inadequate passes or upon failure of puncturing the lesion. The number of passes made and specimen adequacy was not different between the 2 needles. More cross overs from 22 to 25-G needles were observed in uncinate process masses due to technical failures. This study suggested superiority of the 25-G needle in obtaining samples through transduodenal approach.

Fewer studies included 19-G needles for assessment of specimen quality. Sakamoto *et al*[18] included 19-G TCB needle in their study that compared it to 25-G and 22-G needles in 24 patients with solid pancreatic masses. The 22-G and 19-G TCB needles were superior to the 25-G needle ($P < 0.05$) in providing adequate histological diagnosis in technically successful cases. However, the 25-G needle was superior in overall diagnostic accuracy particularly in lesions in the pancreatic head and uncinate process. In another randomized study including 117 patients with pancreatic and peripancreatic masses[61], the traditional 19-G and 22-G needles were compared. Technical failure occurred in 5 out of 60 patients who were randomized to the 19-G needle, all of which arose when sampling pancreatic head or uncinate process lesions. Those masses were successfully sampled once crossed over to 22-G needles. Excluding those with technical failures, the overall diagnostic accuracy was higher in the 19-G group (94.5% *vs* 78.9% P = 0.02). Sample quality was also superior in 19-G needle group (P = 0.03).

Key points: Needle size can influence the quality of samples of EUS-FNA. 22-G and 25-G needles appear to be equivalent in sampling capabilities. 19-G needles appear to be capable of providing superior tissue quantity and quality compared to 22 and 25-G needles, but carry a higher chance of technical failure when utilized for transduodenal sampling.

COMPLICATIONS OF EUS-GUIDED TISSUE ACQUISITION

Complications of EUS-FNA are rare and include bleeding, infection, and acute pancreatitis, which collectively occurs in about 2%-3% or less of procedures[62-63]. Additionally, there have been few case reports of tumor seeding after FNA although this remains very rare[63-67]. Theoretically, the larger the needle size and the higher the number of passes made, the higher the likelihood of complications. Nevertheless, a recent meta-analysis[68] that included 51 studies failed to demonstrate a statistical difference in rate of adverse events associated with 19-G needle as compared to 22 and 25-G needles.

To date, few studies have evaluated the specific factors associated with increased incidence of adverse events. One study of 316 patients[65] found that post-procedural adverse events are higher when accessing pancreatic lesions smaller than 20 mm in size. This can be explained by the fact that smaller lesions can be more difficult to access, and require longer time of penetration and higher number of passes, all of which can increase the risk of damaging the normal surrounding tissues. The same study showed that certain cancers such as neuroendocrine tumors are associated with higher incidence of complications after EUS-FNA, probably due to the highly vascular nature of such lesions. Other variables such as patients' age, needle size, lesion location and number of passes did not have an impact on the incidence of adverse events. It should be noted that due to the very low overall rate of EUS-FNA related complications, a rather large sample size is required to demonstrate any potential increase in complication rates when using larger size needles. In addition, certain complications like bleeding following FNA remain subclinical and rarely result in hospitalizations or further interventions. In general, it is always recommended that the diagnosis be made with smaller needles and minimal number of passes in order to avoid any unnecessary risks.

RECENT ADVANCES IN EUS-GUIDED TISSUE ACQUISITION

The recent technological developments in EUS equipment employ physical concepts of ultrasound in an attempt to improve the diagnostic yield of this procedure while maintaining its high safety profile. One glaring example is the use of real-time sono-elastography, which is a technique that measures tissue elasticity through calculation of tissue strain[69]. Due to the fact that tissue elasticity is often altered when replaced by cancer, EUS elastography can detect small tumors and malignant lymph nodes and this can direct FNA to high yield sites. This can be particularly useful in the setting of chronic pancreatitis, which is estimated to be present in up to 20%-35% of patients undergoing FNA for pancreatic lesions[13,70,71]. This also can be of value when EUS-FNA is negative for malignancy when the suspicion of cancer remains strong[71-75]. Other recent advances such as tridimensional (3D) EUS, contrast-enhanced EUS and EUS spectrum analysis have minimal or no role in tissue acquisition but can provide better visualization of pancreatic masses. This can positively impact the FNA outcome due to better differentiation of malignancies from other inflammatory conditions.

Genetic mutations have been studied as adjunct markers to aid in the diagnosis of pancreatic cancers. The most practical example in relation to FNA of pancreatic cancer is *K-ras* mutation, which has been the focus of several studies to evaluate its impact on the diagnostic yield of FNA[76-78]. *K-ras* oncogene is activated by somatic point substitution and considered as an initial event in pancreatic carcinogenesis[79], and *K-ras* mutation can be found in 90% of patients with this disease. In a recent prospective series including 394 pancreatic masses, Ogura *et al*[76] found that combining *K-ras* mutation analysis with cytopathology increased the sensitivity of EUS-FNA from 87% to 93% ($P < 0.001$) and the accuracy from 89% to 94% ($P < 0.001$) for the diagnosis of pancreatic ductal adenocarcinoma. In this study, out of the 39 patients who were undiagnosed using cytology, K-ras was detected in 18 patients (46%). In a recent meta-analysis[77]. which included 8 studies with 931 patients undergoing EUS-FNA of pancreatic masses, the pooled sensitivity and specificity of EUS-FNA were 80 and 97% respectively; the estimated sensitivity and specificity of *K-ras* mutation analysis were 76.8% and 93.3% respectively, and 88.7% and 92% when cytology and *K-ras* mutation analysis were combined. Overall, *K-ras* mutation testing applied to cases that were inconclusive by EUS-FNA reduced the false-negative rate by 55.6%, with a false-positive rate of 10.7%. In addition to *K-ras* oncogene, a number of tumor suppressor genes were found to be affected by genetic alteration in pancreatic cancer such as p53, p16 and DPC4. Those, in addition to K-ras have been shown to increase the sensitivity of pancreatic cancer detection to up to 90%-100% in cases where FNA was inconclusive[78,79]. Due to the relative high diagnostic accuracy of standard EUS-FNA as well as the relatively high cost and limited availability of these genetic tests, the use of genetic testing of EUS-FNA samples has been limited to research protocols and inconclusive cytopathology specimens.

CONCLUSION

EUS-guided tissue acquisition has evolved to become an indispensible tool for the diagnostic work up of gastrointestinal malignancies and other non-malignant disorders. High sensitivity, specificity and diagnostic accuracy coupled with low rates of adverse events have made this procedure more suitable than other invasive ones such as CT-guided biopsies. The quality of sample acquired by EUS is influenced by numerous factors, including needle size and sampling techniques. Recent studies have demonstrated the adequacy of FNA specimens provided by 25-G needle compared to other needles and this should

be strongly considered in transduodenal sampling. Larger size needles like 19-G appear to provide better sample quality overall, but can be associated with technical failures in the transduodenal approach and potentially higher rates of complications. New flexible 19-G needles and newly designed core biopsy devices appear capable of delivering adequate histopathology samples when this is needed for the diagnosis.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (13): Gastrointestinal endoscopy

Celiac plexus neurolysis in the management of unresectable pancreatic cancer: When and how?

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Abstract

Pancreatic cancer is the second most common abdominal cancer in North America with an estimated 20% resectability at diagnosis, and overall 5-year survival of 5%. Pain is common in pancreatic cancer patients with 70%-80% suffering substantial pain. Celiac plexus neurolysis (CPN) is a technique that can potentially improve pain control in pancreatic cancer while preventing further escalation of opioid consumption. CPN is performed by injecting absolute alcohol into the celiac plexus neural network of ganglia. This review sets out to explore the current status of CPN in non-resectable pancreatic cancer. We will examine: (1) the efficacy and safety of percutaneous-CPN and endoscopic ultrasound guided-CPN; (2) specific technique modifications including bilateral (*vs* central) injections and celiac ganglia neurolysis; and (3) the issue of CPN timing, early at pancreatic cancer diagnosis *vs* traditional late use as salvage therapy.

Key words: Celiac plexus neurolysis; Endoscopic ultrasound; Pancreatic cancer; Pain; Opioid; Gastrointestinal endoscopy

Core tip: The efficacy of salvage celiac plexus neurolysis (CPN) either by percutaneous or endoscopic ultrasound (EUS) guided technique has been modest in its ability to reduce pain and narcotic requirements in patients with unresectable pancreatic cancer, and few studies with rigorous methodology exist. Data for early EUS-CPN at time of diagnosis appears to prevent pain escalation while moderating narcotic use and future studies should explore CPN for patients before rescue therapy is needed. Reports of serious and fatal complications of CPN have surfaced in recent years.

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INTRODUCTION

Pancreatic cancer is the second most common abdominal cancer in North America with an estimated number of 45220 new diagnoses and 38460 deaths in the United States in 2013[1]. The high mortality rate is due in part to the aggressive nature of the tumor and its asymptomatic disease progression leading to delayed diagnosis with an estimated 20% resectability at diagnosis, and overall 5-year survival of 5%[2,3]. Pain is common in pancreatic cancer patients with 70%-80% suffering substantial pain[4-6]. As a result, systemic analgesic therapy (SAT)

usually including opioid medication is central to the management of unresectable pancreatic cancer. However, pain can often become intractable and refractory to narcotics leading to dose escalation and opioid associated side effects[7-9].

Celiac plexus neurolysis (CPN) is a technique that can potentially improve pain control in pancreatic cancer while preventing further escalation of opioid consumption[6,10]. CPN is most often performed by injecting local anesthetic followed by absolute alcohol into the celiac plexus neural network of ganglia with intention to ablate the tissue transmitting pain from the pancreas and adjacent visceral organs. In current clinical practice, it has been used almost exclusively as salvage therapy when pain control is inadequate with SAT[11]. CPN modalities include surgical splanchnectomy, percutaneous (PQ)-CPN, and endoscopic ultrasound guided (EUS)-CPN. Surgical splanchnectomy/intra-operative celiac plexus neurolysis can be performed on those not deemed inoperable preoperatively but will not be reviewed in this paper. The two most commonly practiced routes are the posterior PQ-CPN usually under CT or fluoroscopic guidance and EUS-CPN. There has been much controversy as to which route and which specific techniques should be the gold standard based on efficacy and safety. This is partially due to a lack of well-designed randomized controlled trials and lack of studies directly comparing the two modalities. Furthermore, there is recent data to suggest that using CPN as salvage therapy may not be the only or best option and that early CPN, performed at the time of diagnosis, may prevent or slow the spiral of increasing pain and opioid consumption[12].

This review sets out to explore the current status of CPN in non-resectable pancreatic cancer. We will examine: (1) the efficacy and safety of PQ-CPN and EUS-CPN; (2) specific technique modifications including bilateral (*vs* central) injections and celiac ganglia neurolysis (CGN); and (3) the issue of CPN timing; early at pancreatic cancer diagnosis *vs* traditional late use as salvage therapy.

PQ-CPN

Pain control

Initial meta-analyses regarding the use of PQ-CPN in controlling pain due pancreatic cancer showed conflicting results and are limited to mostly retrospective and uncontrolled studies[10,13,14]. Since then, several RCTs have been published of which 5 (265 patients) from 1993-2008 were analyzed in a recent systematic review[5,15-19]. They demonstrated statistically significant improved pain level in the PQ-CPN group compared to SAT at 1-2 wk by -0.87 [95%CI: -1.47-(-0.28), $P = 0.004$], and at 4 wk by -0.47 [95%CI: -0.71-(-0.23), $P = 0.0001$]. At 8 wk however, the statistical difference was lost -0.31 (95%CI: -0.74-0.12) and similarly no study showed benefit at 12 wk[18]. A previous meta-analysis, by Yan *et al*[6], also comprised of 5 RCTs (302 patients, 3 studies overlap with Nagels *et al*[18]) including one intra-operative neurolysis[5,6,16,17,20,21]. This analysis found pain improvement at 2, 4 and 8 wk of -0.34 (95%CI: -1.03-0.34, $P = 0.33$), -0.50 [95%CI: -0.85-(-0.15), $P = 0.005$], and -0.60 [95%CI: -0.82-(-0.37), $P < 0.00001$] respectively[6].

Regardless of the statistical significance found at different time points between these often heterogeneous studies within 2 meta-analyses, it is striking that all of the point estimates are less than one. A decrease of less than one point on a pain scale is unlikely to be clinically significant and questions whether the procedure is beneficial at all. The difficulty in interpreting the true clinical significance lies in the fact that opioid consumption (see below) is a direct confounder of pain and both pain and opioid use are routinely analyzed with univariate statistical models. If opioid consumption were to simultaneously decrease or even remain unchanged relative to the SAT groups then the difference in pain corrected for opioid use may become clinically significant (data unavailable).

Opioid consumption

To allow for some comparison, data from the 2 above meta-analyses will be used. Nagels *et al*[18] found an absolute reduction in opioid use compared to SAT at 2 wk of -44.64 mg [95%CI: -72.74-(-16.54), $P = 0.002$], 4 wk -72.41 mg [95%CI: -86.14-(-58.68), $P < 0.00001$], 8 wk -70.02 mg [95%CI: -104.05-(-36.00), $P < 0.0001$] and one study at 12 wk (105 ± 65 mg *vs* 169 ± 71 mg, $P < 0.01$)[18]. Yan *et al*[6] found similar findings of decreased opioid use with PQ-CPN at 2 wk -39.99 mg [95%CI: -60.08-(-19.91), $P < 0.0001$], 4 wk -53.69 mg [95%CI: -79.65-(-27.73), $P < 0.0001$] and 8 wk -80.45 mg [95%CI: -134.66-(-26.24), $P = 0.004$].

Some of the above differences in opioid requirements do seem clinically significant, but as mentioned, to measure their true benefit a bivariate or multi-variate analysis would be necessary. These studies also did not convincingly show a decrease benefit in opioid related side effects. However, as discussed below this patient population has symptoms impacted by numerous factors including multiple medications, psycho-social stressors, and mobility. Therefore, to isolate constipation (for example) as strictly a narcotic induced side-effect is likely inappropriate.

Quality of life

Finally, when assessing the effect of PQ-CPN on quality of life (QOL), the data is inconclusive with some studies suggesting an improvement while others failing to demonstrate a significant difference[5,6,15,16,18,19].

It is important to note that the patient population being dealt with are palliative patients at the end of their life. Pain is an extremely complex entity at baseline, and its complexity is only enhanced in patients with a growing and spreading tumor who are facing their own mortality. Although the overall impact on QOL remains controversial, a modest pain reduction in the context of

clinically significant opioid reduction may still be very meaningful. Furthermore, the QOL scales used varied widely and could not be easily combined in any meta-analysis, and the QOL categories themselves within these scales would not be expected to improve by better pain control alone. A simple question such as "did this procedure improve your life in a meaningful way?" may have more appropriately assessed its worthiness. Nevertheless, these concepts and issues still bring into question whether PQ-CPN as a last resort in salvage therapy should be recommended to these patients.

EUS-CPN

EUS-CPN has emerged as a promising approach to CPN that has the potential for better visualization of the celiac plexus through close proximity and real-time high-resolution ultrasound, possibly allowing for more precise and safer injections. However, the data supporting this approach once again in the context of salvage therapy are limited to uncontrolled retrospective studies. Wiersema and Wiersema[22] were the first to describe EUS-CPN in 58 patients and showed modest improvement in pain control up to 12 wk following therapy. More specifically, 45 patients (78%) experienced a decrease in pain score independently of narcotic use. Since then, there have been several other observational studies (with no control group) examining EUS-CPN in relieving pain due to pancreatic cancer[23-26]. In a systematic review of these studies, a significant pain reduction was noted at weeks 2, 4, 8, and 12 with a mean difference in pain score of -4.26 [95%CI: -5.53-(-3.00)], -4.21 [95%CI: -5.29-(-3.13)], -4.13 [95%CI: -4.84-(-3.43)], -4.28 [95%CI: -5.63-(-2.94)] respectively[18]. This is consistent with a meta-analysis, which showed a pain reduction in 80% of the patients following EUS-CPN for pancreatic cancer[27]. EUS-CPN studies showed relatively stable or slightly lower opioid requirements that paralleled this pain reduction[16,17,19]; however, there is no randomized controlled study for EUS-CPN used specifically as salvage therapy despite these promising data.

ADVERSE EVENTS ASSOCIATED WITH CPN

PQ-CPN

It is important to distinguish common or even expected side effects from CPN complications. Frequent minor adverse events associated with PQ-CPN are believed to be due to disturbances of the autonomic system resulting from ablation of the celiac plexus and sympathetic blockade leading to unopposed parasympathetic activity. One study estimated diarrhea (9%), hypotension (8%), constipation (40%), nausea and vomiting (41%), and lethargy (49%)[6]. Pain at the site of injection (96%) has also been frequently reported[10]. Rare complications are described in case reports and include lower neurological deficit (weakness and paresthesia), pneumothorax, and hematuria; and are estimated to occur at 2%[10]. Paraplegia itself is believed to occur secondary to needle trauma or vasospasm induced by the injection of alcohol into the artery of Adamkiewicz leading to ischemic cord injury *via* the anterior spinal artery. Paraplegia has been reported in the literature and is estimated to occur in less than 0.15% of the cases[28].

EUS-CPN

Data on adverse events of EUS-CPN are limited to small retrospective studies and case reports. Similar minor peri-procedural events such as transient hypotension were described in 3 case series and estimated at 11%[23,25,26]. Diarrhea was noted in 4 studies in approximately 18%[23,24,26,29]. Transient abdominal pain was described in case series at rates varying from 1.5% to 8%[22,23,25,26]. Theoretically, EUS might be the safer modality. Its anterior approach through the gastric wall allows for direct passage of the needle into the target area while visualizing and avoiding vascular structures, without having to traverse the retrocrural space near other vital organs. Although initial reports (prior to 2012) of serious adverse events were lacking, there have been a number of severe complications recently reported in the literature. Gimeno-Garcia *et al*[30] reported the first fatal complication with EUS-CPN in the context of chronic pancreatitis leading to celiac artery thrombosis and vasospasm resulting in multi-organ ischemic injury and death. Subsequently, 2 additional reports of ischemic injury and death following EUS-CPN, were also believed to be due to injection of ethanol into the celiac artery leading to vasospasm[31,32]. Other reported complications include retroperitoneal bleeding, and 2 cases of paraplegia[30,33,34].

Overall, although EUS may potentially enhance precision of injections, no conclusions can be made regarding the safer modality without head to head studies with PQ-CPN. Furthermore, serious fatal complications although rare are not unavoidable with EUS-guided therapy.

PQ-CPN *VS* EUS-GUIDED CPN

There are no studies directly comparing EUS-CPN and PQ-CPN in the management of pancreatic cancer. Efficacy of celiac plexus block (CPB) for chronic pancreatitis pain (using an anesthetic agent ± steroids as opposed to ethanol in neurolysis for pancreas cancer) remains controversial. However, two RCTs comparing EUS and PQ-CPN in CPB for chronic pancreatitis have suggested greater efficacy with EUS-CPB than PQ-CPB[35,36]. Gress *et al*[35] studied 20 patients showing greater and more persistent pain relief up to 12 wk post-treatment favoring EUS-CPB. Major weaknesses in this study; however, include its small sample size and unblinded methodology. Santosh *et al*[36] performed a larger, single-blinded RCT involving 56 patients favoring EUS-CPB over PQ-CPB for initial pain relief with 70% *vs* 30% responding to treatment respectively. Pain relief was also shown to be

more persistent with 38% *vs* 10% having significant pain relief at 12 wk. Although data from these RCTs of CPB in chronic pancreatitis are not directly applicable to CPN in pancreatic cancer, they do suggest a potential superior efficacy with they do suggest a potential superior efficacy with EUS in terms of drug delivery. Trials comparing PQ and EUS-CPN are certainly needed.

BILATERAL OR UNILATERAL CPN AND CGN

Bilateral vs unilateral/central neurolysis

Unilateral neurolysis is accomplished by a single injection into the base of the celiac artery takeoff. This technique may not adequately expose celiac ganglia to ethanol as it is now appreciated that the majority of ganglia are between the celiac artery and left adrenal gland[37].

Bilateral injection, is performed by injecting into both sides of the celiac plexus by torqueing the echoendoscope to each side of the celiac artery and advancing the injection needle parallel to its trajectory. Although there is potential for more adverse events due to greater needle movement, the bilateral approach has been shown by some to be more effective in providing pain relief. In a prospective cohort study comparing unilateral *vs* bilateral CPN or CPB, the bilateral technique achieved significantly more pain relief *vs* unilateral (mean percent pain reduction) 70.4% (61.0, 80.0) *vs* 45.9% (32.7, 57.4), P = 0.0016, at day 7 post treatment. Although this is a short-term study the onset of neurolysis effect begins soon after the nerve ablation, therefore a comparison between two techniques at 7 d can still be revealing. The only predictor of a > 50% pain reduction was bilateral injection [odds ratio 3.55, (95%CI: 1.72-7.34)][38]. Furthermore a meta-analysis also suggested superiority of pain reduction with bilateral injection over central injection, 85.54% *vs* 45.99% respectively[27]. A subsequent RCT comparing the two approaches in pancreatic cancer in 50 patients did not suggest a significant difference in terms of pain control or adverse events. However, there was a trend to nearly a 30% increase in duration of effect (11 wk *vs* 14 wk) in favor of bilateral, a result, which may have been limited by sample size[39]. Furthermore, the point estimate for central/unilateral seemed high at 69% (compared to approximately 45% in other studies) and may have prevented a difference from being detected. One must keep in mind that meta-analyses of the highest quality studies for both PQ-CPN and EUS-CPN included almost only the bilateral technique[6,27]. The bilateral technique also requires significant advancement on either side of the celiac artery and may be operator dependent. Finally, two other studies also support the notion that injection deeper[40] and along both sides of the celiac axis provide better pain relief[41]. Although there is no definitively proven superior technique, we favor the bilateral technique given the sum of the above evidence as well as the concept of wider distribution of the ethanol near areas where ganglia are most commonly found.

Central ganglia neurolysis

Recent developments in EUS equipment have improved resolution such that injection directly into celiac ganglia is possible in certain patients. One prospective study in 200 patients undergoing diagnostic EUS demonstrated a rate of celiac ganglia detection of 81%, Figure 1[42]. Another study demonstrated a ganglia visualization rate of 89% in 57 patients[43]. These percentages seem high in our experience nevertheless exemplify the real possibility of visualizing ganglia. Since ganglia are collections of nerve bodies and glial cells, injections into these structures have the potential to obliterate more neurons successfully, possibly leading to greater pain suppression. Levy *et al*[44] provided preliminary data on EUS-CGN demonstrating its safety and effectiveness in achieving significant pain relief in 94% of the subjects with pancreatic cancer. In addition, a retrospective analysis of EUS-CPN and CGN found that visualization of celiac ganglia was the best predictor of response to therapy[26]. Recently, an RCT comparing EUS-CGN *vs* EUS unilateral CPN showed substantial greater pain relief in the CGN group (73.5% *vs* 45.5%, P = 0.026) with similar adverse events[37]. However, the comparison was not against bilateral injection and the response rate with CGN *vs* unilateral was remarkably similar to the bilateral *vs* unilateral technique referenced above 70.4% (61.0, 80.0) *vs* 45.9% (32.7, 57.4), P = 0.0016[38].

Overall, superior efficacy of EUS-CGN is possible but unproven, especially compared to bilateral injection. This also lends biologic plausibility to bilateral injection being more efficacious than central since the ganglia frequently "and remove" as very are located lateral to the celiac artery and may be injected with the bilateral technique even when not visualized. CGN is also not possible *via* PQ-CPN. With EUS-guided CGN, although the drug is injected into the ganglia, it is conceivable that drug also diffuses beyond the targeted ganglia and destroys adjacent, invisible ganglia. Also, variation in equipment, make and model significantly impact ability to visualize ganglia and so success rates cannot be generalized. At this time we do not recommend CGN as a standard for CPN technique as it does not provide a wider distribution of the ethanol over the bilateral technique, but does add a degree of technical complexity and dependence on quality of equipment.

TIMING OF CPN: EARLY (NEAR TIME OF DIAGNOSIS) *VS* TRADITIONAL SALVAGE THERAPY

We hypothesize that one of the primary reasons why the magnitude of effect shown for salvage CPN is often seen as marginal and not clearly clinically meaningful is that it is offered "too late". Once pancreatic cancer has progressed causing increasing pain and tolerance to narcotics, a true rescue is unlikely to occur. The postulated advantage of early therapy therefore is to prevent

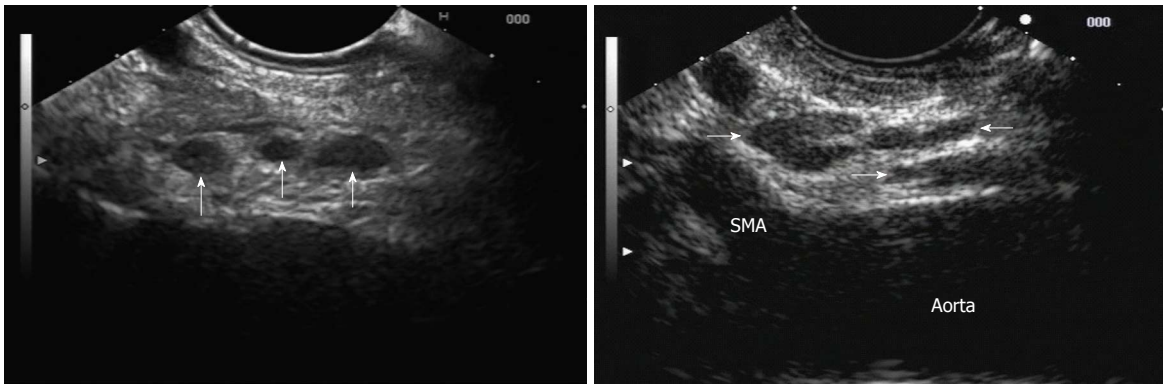

Figure 1 Three celiac ganglia are demonstrated in each image (arrows). SMA: Superior mesenteric artery.

or minimize both pain progression and narcotic dose escalation and tolerance. We addressed this issue in the first sham controlled RCT comparing early EUS-CPN (at the time of diagnosis) for unresectable pancreatic cancer *vs* standard SAT[12]. The difference in absolute mean change in pain between the early and salvage therapies were -1.0 [95%CI: -1.7-(-0.1)] at 1 mo and -2.2 [95%CI: -3.1-(-1.4)] at 3 mo favoring the early CPN group. Despite starting with a lower pain level than salvage therapy trials the absolute decrease in pain was greater than those found in the PQ-CPN RCTs above, and statistically significant at 3 mo. For difference in mean percent change in pain score the EUS-CPN group trended at 1 mo and was significantly greater at 3 mo as well, -28.9% (95%CI: -67.0-2.8, P = 0.09), and -60.7% [95%CI: -86.6-(-25.5), P = 0.01] respectively. In the SAT group, morphine use increased compared with baseline at both 1 mo (mean absolute change in MEQ consumption +54 mg [95%CI: +20-(+96)] and particularly at 3 mo (mean absolute change in MEQ consumption +100 mg [95%CI: +49-(+180)]. In the EUS-CPN group, morphine use also increased at 1 mo (mean change in MEQ consumption +53 mg [95%CI: +28-(+89)], but plateaued by 3 mo (mean change in MEQ consumption +50 mg [95%CI: +28-(+79)]. Comparing groups, EUS-CPN did not significantly reduce narcotic use at 1 mo, however, at 3 mo post-procedure there was a strong trend towards lower opioid consumption in the CPN group -49.5 mg (95%CI: -127.5-7.0, P = 0.10). Importantly, patients who did not receive subsequent radiation or chemotherapy demonstrated greater difference between groups. For example, a significant reduction in narcotic consumption was noted at 3 mo -144.5 mg [95%CI: -290.0-(-30.0)] (Table 1). The stronger results in patients who did not undergo adjuvant therapy underlies that this therapy with its inherent benefit to the patient, diluted the magnitude of effect of CPN alone. Therefore, data from this RCT suggest that early EUS-CPN prevents pain escalation while moderating narcotic use. Compared to all of the studies using salvage therapy, both PQ and EUS-CPN, these results seem very favorable.

CONCLUSION

Severe and intractable pain refractory to traditional SAT is a common occurrence of non-resectable pancreatic cancer. Pain control is crucial in the management of this population with several retrospective studies and a handful of RCTs demonstrating greater pain relief with equal and/or decreased opioid requirements with CPN (PQ or EUS). Although there are no head to head trials comparing EUS to PQ-CPN, data comparing the two modalities for CPB in chronic pancreatitis suggests EUS may be superior. Despite no conclusive data suggesting superiority, EUS does offer the potential for enhanced visualization of important vital structures and of celiac ganglia should CGN studies become more robust. Given the sum of the evidence and with wider distribution of ethanol in areas where ganglia are known to reside, we favor bilateral CPN over central injection. However this superiority is still controversial and central injections are certainly acceptable if the echoendoscopist is more comfortable with the latter. CGN cannot yet be recommended given inconsistent visualization of ganglia and the lack of trials compared to the bilateral technique which itself can be reproduced consistently in patients using only the celiac artery as a landmark. Perhaps most importantly, we feel there should be an emphasis of future studies on performing CPN early (at or near diagnosis) and the only existing EUS-CPN RCT did examine this approach with results comparable and seemingly superior to existing PQ-CPN RCTs done exclusively for salvage therapy. Preventing the escalation of pain and narcotic use should be the purpose of CPN in patients with unresectable pancreatic cancer. One must note, however, that pain due to pancreatic cancer is multifactorial not only including celiac plexus pathways but also from, for example, intestinal obstruction and liver capsule distention from metastases. CPN will only target some of these pain mechanisms and may play less of a role as disease progresses and other pain etiologies become more pronounced.

Given the totality of existing evidence, it appears that in 2013, the optimal patient for successful and meaningful CPN would be undergoing diagnostic and staging

Table 1 Early endoscopic ultrasound-celiac plexus neurolysis *vs* systemic analgesic therapy: Pain relief and narcotic consumption with or without chemo-XRT[12]

EUS-CPN *vs* control	After 1 mo (95%CI)	After 3 mo (95%CI)
Difference in mean % change in pain relief:		
No chemo-XRT[1]	-59.6% (-95.4 to -27.6)[1]	-85.8% (-127.6 to -51.3)[1]
Chemo-XRT	31.0% (-34.3 to 106.2)	-45.6% (-72.6 to -23.3)[1]
Difference in mean change in MEQ consumption:		
No chemo-XRT	-2.4 mg (-58.4 to 60.8)	-144.5 mg (-291 to -30)[1]
Chemo-XRT	11.4 mg (-23.7 to 39.4)	26.1 mg (-12.2 to 56.5)

[1]Statistically significant $P < 0.05$. EUS-CPN: Endoscopic ultrasound-celiac plexus neurolysis; Chemo-XRT: Chemotherapy and radiation therapy; MEQ: Morphine equivalent.

EUS for pancreas cancer. CPN in this instance may be more impactful if the patient happens to not undergo subsequent chemotherapy/radiotherapy. Rare, serious, and even life threatening complications regardless of timing and route have to be disclosed and discussed with the patient in detail. Future studies should focus on early CPN in this unfortunate patient population.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (13): Gastrointestinal endoscopy

Is the type of insufflation a key issue in gastro-intestinal endoscopy?

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Abstract

Endoscopic procedures continue to play an emerging role in diagnosing and treating upper and lower gastrointestinal (GI) disorders. In particular, the introduction of colonoscopy in bowel cancer screening has underlined its promising role in decreasing the incidence of colorectal cancer and reducing tumour related mortality. To achieve these goals patients need to contemplate endoscopic examinations as painless and fearless procedures. The use of carbon dioxide (CO_2) as an alternative insufflation gas in comparison to air has been considered as an essential key to improving patients' acceptance in undergoing endoscopic procedures. CO_2 is absorbed quickly through the bowel mucosa causing less luminal distension and potentially less abdominal pain. However, its exact role has not been defined completely. In particular, the beneficial use of CO_2 in upper GI endoscopy and in sedated patients is still conflicting. In the present review, we aimed to assess the current evidence for using CO_2 in endoscopy and to evaluate its potential role in the future.

Key words: Gastrointestinal endoscopy; Colonoscopy; Endoscopic retrograde cholangiopancreatography; Insufflation gas; Carbon dioxide

Core tip: With the increasing use of gastrointestinal endoscopy, especially for screening in an asymptomatic population, increasing the tolerability of the procedure is of paramount importance. Our review summarizes evidence that carbon dioxide (CO_2) insufflation can reduce both pain and bloating in colonoscopy and endoscopic retrograde cholangiopancreatography although the evidence in gastroscopy is still lacking. Despite established safety concerns about hypercapnia, significant harm has never been demonstrated in the literature. Patients thought to be at higher risk of hypercapnia need to be included in more studies to demonstrate that CO_2 insufflation is safe in an unselected screening population but early evidence is encouraging.

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INTRODUCTION

In the last decade, endoscopy has become an essential diagnostic and therapeutic instrument in daily clinical practice. As a consequence, the number of endoscopic examinations has increased continuously, in particular, as a result of constant efforts to improve patient's acceptance and compliance to participate in bowel cancer screening programs. However, some patients still have a fear of undergoing colonoscopy, as they associate it with

considerable pain and discomfort.

A number of studies aimed to investigate how to ease abdominal symptoms in lower and upper gastrointestinal (GI) endoscopy[1]. The introduction of moderate or deep sedation has certainly been an essential step to increase its attractiveness and to reduce the anxiety and concerns of the patients[2]. Recent evidence demonstrates that sedation can be safely administered in colonoscopy without increasing the risk of respiratory or abdominal complications[3].

Another technique that has emerged in the last few years is the use of carbon dioxide (CO_2) as an alternative insufflation gas. CO_2 is rapidly absorbed by the intestinal mucosa and easily expired through the respiratory tract, with the potential advantage of reducing the duration of large bowel distension. However, there have also been concerns as whether CO_2 results in a raise in arterial pressure of CO_2 (pCO_2) leading to cardiac or respiratory compromise[4].

Notably, several studies revealed promising results with significantly less abdominal pain during and after endoscopic procedures by using CO_2 compared to air insufflation, which is still considered the standard gas to insufflate the bowel[5-7]. In addition, in upper GI endoscopy the use of CO_2 gas remains conflicting in the current literature and convincing evidence is still missing to warrants its routine use[8]. It is also debatable whether the use of CO_2 is still beneficial in patients, who are deeply sedated during the procedure.

In the present review, we aimed to assess the current evidence for the use of CO_2 insufflation during diagnostic and therapeutic endoscopic procedures and to define its role in the future.

LOWER GI ENDOSCOPY

A high number randomised controlled trials comparing endoscopic insufflation with either CO_2 or air were conducted in the last decade (Table 1). Interestingly, no studies to date have noted any technical disadvantages when using CO_2 insufflation; insertion and withdrawal times, caecal intubation rates and complication rates are comparable or even superior in favour of CO_2[9]. The volume of gas used has also been compared in several studies and no difference has been found when using CO_2 compared to air[10].

The primary outcome measure in the majority of studies was pain, as measured using a visual analogue scale (VAS). Findings have consistently shown lower pain scores when CO_2 insufflation was used in contrast to air, although some studies have shown a peak difference in pain score during the procedure or shortly after whereas others have shown evidence of a more delayed effect several hours after the procedure[7,11,12]. There is considerable heterogeneity between studies in the time intervals at which pain was measured.

Several studies have attempted to assess the degree of abdominal bloating objectively post procedure by assessing either the degree of colonic distension present on abdominal radiograph or the changes in waist circumference post procedure[7,11,13,14]. Findings have consistently demonstrated less distension in the group undergoing CO_2 insufflation. The differences were marked with very little overlap between groups. For example Sumanac found that 71% of patients had large bowel dilatation of > 6 cm 1 h after colonoscopy with air insufflation compared with only 4% in the CO_2 group[7].

Iida *et al*[15] investigated patients having CO_2 compared to air insufflation colonoscopies and measured their levels of salivary alpha-amylase (SAA) as an objective maker of stress[15]. SAA levels increased as a result of colonoscopy in both groups as expected but the rise was significantly higher in the air group than the CO_2 group. VAS scores were also measured however and there was no significant difference between the groups.

Sedation

Available studies used a wide range of sedation methods from no sedation to deep sedation with agents such as propofol. There has been some question as to whether the potential benefits of reduced pain with CO_2 insufflation may be lost when deep sedation is used. The evidence would point to the contrary however with the majority of studies showing benefits lasting beyond the time that the sedation would have worn off[5,7,11,16,17]. Riss *et al*[5] used deep sedation and observed the greatest improvement in pain scores between 15 min and 6 h post-procedure showing that there is still a potential benefit.

Effects on screening

Making colonoscopy more comfortable is an issue of particular concern when considering bowel cancer screening programmes where asymptomatic patients are voluntarily undergoing the procedure with no guaranteed benefit. Several studies have addressed patients' feelings about undergoing further colonoscopies when comparing air and CO_2 insufflation to determine whether a more comfortable procedure would increase compliance with ongoing screening. The results showed a high level of satisfaction with the procedure, with the vast majority of patients reporting that they would be happy to go ahead with a repeat colonoscopy if necessary and would recommend it to others[5,12]. Geyer *et al*[17] found that overall satisfaction was slightly higher in the CO_2 group (9.6 *vs* 9.3 on a VAS) however other studies have found no significant difference between the air and CO_2 groups[5,12,17]. This does not strongly support the hypothesis that the use of CO_2 could improve compliance with screening programmes.

CO_2 insufflation only during scope withdrawal

There has been recent interest in whether using CO_2 insufflation only for the withdrawal phase of colonoscopy retains the same benefits of reduced pain and distension as when CO_2 is used for the entire procedure. Chen *et al*[18] and Hsu *et al*[19] both found that there was no difference in

Table 1 Studies comparing carbon dioxide and air insufflation in colonoscopy

Ref.	Patients (*n*)	Exclusion criteria	Sedation	Findings
Stevenson *et al*[11], 1992	56	Previous colonic resections, children	Moderate	No difference in pain during procedure but better in CO_2 group at 6 and 24 h post AXR at 1 h - 94% trace/v little gas in CO_2 group, air 18% > 10 cm, 57% > 6 cm
Sumanac *et al*[7], 2002	100	GI bleed, IBD, colectomy	Moderate	AXR at 1 h - 71% > 6 cm in air *vs* 4% in CO_2 group Reduced pain score at 1 and 6 h in CO_2 group
Bretthauer *et al*[16], 2002	240	Previous resection, malignancy, severe cardiac or respiratory disease	None	Lower pain score at all time points in CO_2 group No difference in $ETCO_2$
Church *et al*[33], 2003	247	None	Moderate	Lower pain score 10 min post procedure in CO_2 group but no difference during
Bretthauer *et al*[34], 2005	103	Severe COPD, children	Moderate /none	Lower pain in CO_2 group at 1, 3, and 6 h Higher $ETCO_2$ in both groups when sedated
Wong *et al*[12], 2008	96	COPD, colectomy, bleeding, obstruction	Moderate	Lower pain score in CO_2 group during procedure and in first 30 min then no difference 93% CO_2 *vs* 98% air would have procedure again 89% CO_2 *vs* 96% air would recommend to others
Liu *et al*[35], 2009	349	None	None	Lower pain score in CO_2 group No difference in $ETCO_2$
Riss *et al*[5], 2009	300	Severe COPD, children	Deep	Lower pain score in CO_2 group at 15 m, 30 m and 6 h but not during procedure 98% overall would have procedure again, no difference between groups
Geyer *et al*[17], 2011	109	None	Moderate/ deep	Less pain and bloating (peak at 1 h) in CO_2 group No change in $TCCO_2$
Yamano *et al*[27], 2010	120	Previous resection, malignancy, severe cardiac or respiratory disease, active bleeding, obstuction	None	Lower pain score in CO_2 group
Mayr *et al*[36], 2012	156	None	Moderate	No rise in $TCCO_2$ No pain in 84% of CO_2 group *vs* 65% of air group
Singh *et al*[6], 2012	142	Previous resection	Deep	Higher caecal intubation rate and faster in CO_2 group Less discomfort in CO_2 group
Díez-Redondo *et al*[37], 2012	282	None	Moderate/ deep	Reduced pain scores in CO_2 group for first 6 h
Chen *et al*[18], 2013	193	None	None	No difference in VAS
Iida *et al*[15], 2013	100	None	Moderate	Reduced salivary stress hormones in CO_2 group No difference in VAS score
Uraoka *et al*[38], 2009	114	Easy colonoscopies	None	Overall lower pain in CO_2 group, particularly when done by less experienced endoscopists
Fernández-Calderón *et al*[13], 2012	214	None	Deep	Lower pain in CO_2 group Greater increase in waist circumference in air group
Seo *et al*[14], 2013	94	None	Moderate	Less pain in CO_2 group Greater increase in waist circumference in air group

CO_2: Carbon dioxide; AXR: Abdominal radiograph; GI: Gastrointestinal; IBD: Inflammatory bowel disease; $ETCO_2$: End-tidal carbon dioxide; COPD: Chronic obstructive pulmonary disease; $TCCO_2$: Transcutaneous carbon dioxide; VAS: Visual analogue score.

pain score when CO_2 was used only for withdrawal[18,19].

Given that there seem to be no proven disadvantages in using CO_2 for the entire procedure it is unclear what advantage would be offered by using air for insertion then changing to CO_2 mid-procedure. One would assume that using two insufflation systems for each patient would have negative implications in terms of both time and cost.

UPPER GI ENDOSCOPY, ENDOSCOPIC RETROGRADE CHOLANGIOPANCREATOGRAPHY AND ENDOSCOPIC RESECTION PROCEDURES

There is increasing interest in the use of CO_2 insufflation in gastric and oesophageal endoscopic submucosal resection procedures as well as endoscopic retrograde cholangiopancreatography (ERCP) where lengthy procedures may be necessary and abdominal pain from small bowel distension may be significant. There have been no studies looking at CO_2 insufflation solely for gastroscopy without endoscopic surgery, ERCP or consecutive colonoscopy, presumably because post procedural pain is less of a problem than with colonoscopy.

A meta-analysis of 7 high quality RCTs (including a total of 756 patients) comparing CO_2 to air insufflation in ERCP was carried out by Shi *et al*[20]. The authors found that there was a significant reduction in abdominal pain at 1, 3 and 6 h post procedure when CO_2 was used although at 24 h there was no significant difference. There was no difference in the procedure time but a

Table 2 Summary of studies comparing the type of insufflation in endoscopic retrograde cholangiopancreatography

Ref.	Patients (*n*)	Exclusion criteria	Sedation	Findings
Bretthauer *et al*[29], 2007	118	COPD with known CO_2 retention	Moderate	Less pain up to 24 h in CO_2 group Increased $TCCO_2$ equally in both groups while under sedation
Maple *et al*[39], 2009	105	COPD, pre-procedure abdominal pain	Deep	Less pain at 1 h CO_2 group, no difference at 24 h
Dellon *et al*[30], 2010	78	COPD on home O_2, known CO_2 retention or opiate use	Moderate	Fewer adverse events in CO_2 group No difference in pain scores Increased $TCCO_2$ equally in both groups while under sedation
Kuwatani *et al*[40], 2011	80	COPD, pre-procedure abdominal pain	Deep	No difference in pain scores
Luigiano *et al*[31], 2011	110	COPD, pre-procedure abdominal pain	General anaesthesia	Less pain at 1, 3 and 6 h in CO_2 group, no difference at 24 h Higher $TCCO_2$ in CO_2 group but easily compensated for with hyperventilation
Muraki *et al*[23], 2012	208	COPD	Deep	Less evidence of physiological stress in CO_2 group Borderline lower complications in CO_2 group

COPD: Chronic obstructive pulmonary disease; CO_2: Carbon dioxide; $TCCO_2$: Transcutaneous carbon dioxide.

borderline reduction in complications was found in the CO_2 group (pooled OR = 0.51; 95%CI: 0.27-0.97, P = 0.04). Further similar meta-analyses have been carried out by Cheng *et al*[21] and Wu *et al*[22] (Table 2) with similar findings[21,22]. There may be particular advantages for less experienced endoscopists when using CO_2 insufflation as small bowel distension can make the procedure technically more difficult: Muraki *et al*[23] used physiological parameters and complications as outcome measures when ERCP was being carried out by non-expert endoscopists and found CO_2 insufflation resulted in less physiological stress and borderline lower complications when compared to air.

Only a small number of studies have investigated the use of CO_2 insufflation for endoscopic resection procedures so far. The majority have concentrated on safety rather than pain scores[24,25]. Maeda *et al*[26] found that there was less gas present in the GI tract (assessed on CT scan) after CO_2 insufflation but no difference in VAS scores or complication rates.

SAFETY CONCERNS

There have been established concerns that the use of CO_2 insufflation may increase the systemic partial pCO_2 and put strain on the respiratory system in trying to eliminate this. Hypercapnia can have a range of physiological effects in addition to respiratory stimulation including direct and indirect effects (*via* stimulation of the sympathetic nervous system). Predominantly the effects are cardiovascular, including peripheral vasoconstriction and tachycardia, and neurological, including confusion and reduced consciousness. For this reason the majority of RCTs have excluded large groups of patients such as those with cardiac or respiratory disease, those taking opiate analgesia and those known to have high baseline pCO_2 levels. Several studies have attempted to quantify the effects on blood CO_2 by measuring this either transcutaneously, with end-tidal CO_2 or blood sampling[16,27].

Bretthauer found no difference in $ETCO_2$ in unsedated patient undergoing colonoscopy, in fact CO_2 levels fell during the procedure in both groups[16]. In patients undergoing sedated colonoscopy, particularly in deep sedation, an increase in CO_2 has been found during the procedure but this was equally true for both air and CO_2 groups and was likely to be due to respiratory depression due to sedation rather than the reabsorption of CO_2 from the colon[28]. One potential limitation of many of these studies is the unreliability of indirect CO_2 measurement with transcutaneous or end tidal CO_2 measurement. Serial arterial blood gases may be more accurate but it was felt that this would be unacceptable to patients and therefore has not been widely used in studies so far.

For ERCP, safety data was analyzed in three of the RCTs. In two studies using sedation there was no difference in pCO_2 between the two groups but in a single study which carried out ERCP under general anaesthetic with endotracheal intubation there were significantly higher pCO_2 levels in the CO_2 insufflation group, although this was easily compensated for by hyperventilation[29-31]. All RCTs excluded patients with COPD although some only excluded patients with severe COPD evidenced by known CO_2 retention or use of home oxygen. The rise in CO_2 in patients having ERCP under general anaesthetic may be of concern as it implies that patients probably hyperventilate to some degree to remove the extra CO_2. When they were anaesthetized this didn't happen and CO_2 rose. In patients with significant respiratory disease it may be that they are not able to cope with this compensation but only small numbers of patients have been studied so far.

Suzuki *et al*[24] monitored the arterial pCO_2 in 100 patients undergoing prolonged CO_2 insufflation for endoscopic submucosal dissection under general anaesthesia and found that although pCO_2 rose to a median peak of 39 mmHg, this was acceptable and easily controllable and there was little correlation with procedure time. There was no air group for comparison. Takano *et al*[25]

carried out a crossover trial and found no difference in pCO_2 when air or CO_2 insufflation was being used.

So far the majority of studies have found no significant increase in pCO_2 in patients undergoing endoscopy with CO_2 insufflation compared to air insufflation. Although CO_2 insufflation has been shown to be safe in all studies to date, the exclusion of patients with respiratory disease in many studies means that these results cannot be applied to all patient groups and the participants are not representative of a screening population. Later studies have addressed this by removing any exclusion criteria. Geyer found that there was no significant rise above normal CO_2 levels in an unselected population[17]. Changing over to use CO_2 as standard for endoscopy would mean using this in high risk groups where less safety data is available but there is no evidence so far to suggest that exclusion of particular patient groups is necessary.

Current sedation and monitoring guidelines are summarized by Lichtenstein *et al*[32]. They advocate the use of opiates and benzodiazepine for moderate sedation and monitoring with clinical observation, pulse oximetry and non-invasive blood pressure measurement. The use of capnography or other advanced monitoring was not advocated for patients undergoing moderate sedation. In "low risk" patients as included in most studies there were not significant problems with hypercapnia therefore this level of monitoring is likely to be adequate. In "high risk" patients more at risk of hypercapnia or respiratory complications further monitoring could be considered due to the current paucity of evidence in this population.

COST ANALYSIS

Yamano *et al*[27] estimated that the use of CO_2 in their unit increased the cost of each colonoscopy by 2.5%. This cost estimation was related to the gas used and the initial cost of a CO_2 insufflation system also needs to be taken into account by units considering changing to CO_2 rather than air insufflation. This may be offset in the longer term by less use of sedation, potentially shorter stays following the procedure and a lower readmission rate.

A cost analysis was carried out as part of the meta-analysis by Cheng *et al*[21] comparing air and CO_2 insufflation in ERCP. They analysed equipment, hospital, radiology and physician costs and found no significant cost difference between the two methods.

CONCLUSION

In the light of available RCT´s and subsequent meta-analyses, several conclusions can be drawn with potential clinical relevance. The use of CO_2 in colonoscopy has significant advantages compared to air insufflation. Especially, abdominal pain and bloating during and after the procedure were reduced in the CO_2 insufflation group in the vast majority of published studies. Notably, this positive effect was also detectable in patients, who were deeply sedated during endoscopy. The question of whether CO_2 insufflation results in improved patient satisfaction was found to be controversial, however, it is assumable that patients with less pain also tend to repeat or recommend colonoscopy more likely. The concern that CO_2 increases the risk of complications due to elevated systemic partial pressure of CO_2 has not been studied intensively, but recent data support its widespread use in an unselected population.

In contrast, the use of CO_2 in upper GI endoscopy is not clearly defined and further well designed studies are mandatory to assess it exact role in this field.

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TOPIC HIGHLIGHT

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Endoscopic innovations to increase the adenoma detection rate during colonoscopy

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Abstract

Up to a quarter of polyps and adenomas are missed during colonoscopy due to poor visualization behind folds and the inner curves of flexures, and the presence of flat lesions that are difficult to detect. These numbers may however be conservative because they mainly come from back-to-back studies performed with standard colonoscopes, which are unable to visualize the entire mucosal surface. In the past several years, new endoscopic techniques have been introduced to improve the detection of polyps and adenomas. The introduction of high definition colonoscopes and visual image enhancement technologies have been suggested to lead to better recognition of flat and small lesions, but the absolute increase in diagnostic yield seems limited. Cap assisted colonoscopy and water-exchange colonoscopy are methods to facilitate cecal intubation and increase patients comfort, but show only a marginal or no benefit on polyp and adenoma detection. Retroflexion is routinely used in the rectum for the inspection of the dentate line, but withdrawal in retroflexion in the colon is in general not recommended due to the risk of perforation. In contrast, colonoscopy with the Third-Eye Retroscope® may result in considerable lower miss rates compared to standard colonoscopy, but this technique is not practical in case of polypectomy and is more time consuming. The recently introduced Full Spectrum Endoscopy™ colonoscopes maintains the technical capabilities of standard colonoscopes and provides a much wider view of 330 degrees compared to the 170 degrees with standard colonoscopes. Remarkable lower adenoma miss rates with this new technique were recently demonstrated in the first randomized study. Nonetheless, more studies are required to determine the exact additional diagnostic yield in clinical practice. Optimizing the efficacy of colorectal cancer screening and surveillance requires high definition colonoscopes with improved virtual chromoendoscopy technology that visualize the whole colon mucosa while maintaining optimal washing, suction and therapeutic capabilities, and keeping the procedural time as low and patient discomfort as optimal as possible.

Key words: Colonoscopy; Endoscopic innovations; Adenoma detection; Polyp detection; Gastrointestinal endoscopy

Core tip: Up to a quarter of polyps and adenomas are missed during colonoscopy due to poor visualization behind folds and the inner curves of flexures, and the presence of flat lesions that are difficult to detect. In the past several years, new endoscopic techniques have been introduced to improve the detection of polyps and adenomas. Optimizing the efficacy of colorectal cancer screening and surveillance requires high definition colonoscopes with improved virtual chromoendoscopy technology that visualize the whole colon mucosa while maintaining optimal washing, suction and therapeutic capabilities, and keeping the proce-

dural time as low and patient discomfort as optimal as possible.

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INTRODUCTION

Colonoscopy is considered the gold standard for the detection and removal of polyps and adenomas in the colorectum. There is strong evidence that the removal of polyps and adenomas by colonoscopy lowers colorectal cancer (CRC) incidence and mortality[1,2]. However, in recent years there has been an increasing concern about the effectiveness of colonoscopy the detection of adenomas, early-stage CRC and especially right-sided cancers[3]. Population-based studies have reported that 3%-8% of patients with CRC had a colonoscopy within 3-5 years prior to CRC diagnosis[4-6]. Retrospective studies revealed that these so-called interval or post-colonoscopy cancers can mainly be attributed to missed lesions or inadequate examination[5]. Indeed, a considerable proportion of polyps and adenomas are being missed with colonoscopy, with overall polyp and adenoma miss rates being estimated between 20%-25% in most back-to-back colonoscopy studies[7].

The main factors thought to be responsible for missing lesions, besides endoscopist dependent factors, include the relative difficulty to visualize polyps at the proximal side of haustral folds and internal curves of flexures[8,9], the presence of flat lesions[10] and poor bowel preparation[11]. In addition, especially right-sided advanced adenomas are more often diminutive in size or non-polypoid in appearance compared to left-sided advanced adenomas and may therefore be more easily overlooked[10,12]. Surface visualization with standard 140 and 170 degrees colonoscopes is approximately between 87% and 92% in a clean colon, which illustrates the limitation of standard colonoscopes to adequately visualize the entire mucosa[13]. As a result, premalignant lesions can be missed and it been shown that two-thirds of the non-rectal $\geqslant$ 6 mm lesions that are missed during colonoscopy are located on the proximal side of folds[9].

In more recent years, new endoscopic technologies aimed to increase polyp detection rates (PDR) and adenoma detection rate (ADR) have been developed. In this review we will discuss these endoscopic innovations and their potential to improve the detection of premalignant lesions during colonoscopy (Table 1).

HIGH-DEFINITION COLONOSCOPY

High-definition colonoscopy uses a high definition monitor that enables more images per second to be shown. Moreover, the images have a higher resolution as compared to standard definition colonoscopy. Although high definition colonoscopy provides much better imaging, studies evaluating polyp detection with high definition as compared to standard definition colonoscopes are scarce and show conflicting results[14]. Two randomized trials[15,16] found no significant differences in ADR and PDR between both techniques. In contrast, one randomized study reported a higher PDR (64% *vs* 53%, $P = 0.03$) and mean number of small hyperplastic polyps per subject (0.10 *vs* 0.25, $P = 0.003$) with high definition colonoscopy[17], while in another randomized multicenter study[18] high definition colonoscopy yielded more adenomas per subject (1.12 *vs* 0.69, $P = 0.02$) and especially flat adenomas and right-sided adenomas (both $P < 0.01$). Furthermore, East *et al*[19] reported in a prospective non-randomized study more diminutive (< 6 mm), non-flat adenomas with high definition colonoscopy, although no significant differences in ADR and PDR could be demonstrated. Similar results were found in a retrospective study by Buchner *et al*[20] including 1226 patients undergoing standard definition colonoscopy and 1204 patients undergoing high definition colonoscopy. Both ADR (28.8% *vs* 24.3%) and PDR (42.2% *vs* 37.8%) were statistically significantly higher with high definition colonoscopy but this mainly concerned smaller lesions.

Hence, the use of high definition colonoscopy leads to high quality images and a marginal increase in ADR and PDR compared to standard definition colonoscopy. The absolute increase in ADR is however small and is estimated to be approximately 3.5% according to a meta-analysis with pooled data of five studies in 4422 patients[21]. The additional value of high definition colonoscopy seems mainly limited to small lesions and, according to one study, flat lesions in the right colon. However, caution is required when interpreting the results because marked heterogeneity exists with differences in study design and the type of population included.

VIRTUAL CHROMOENDOSCOPY

Virtual chromoendoscopy uses a narrow spectrum of wavelengths with a decreased penetration depth to enhance visualization of the colon mucosa and has been developed as an alternative to dye assisted chromoendoscopy. Light of short wavelengths increases the vascular contrast of the mucosa, allowing improved visualization of the colonic mucosal surface. Manufactures have developed multiple techniques including Narrow Band Imaging (NBI), Fuji Intelligent Color Enhancement (FICE) and Autofluorescence Imaging (AFI), which can easily be switched on during colonoscopy. These techniques have been suggested to improve the detection of (subtle) mucosal lesions[22-24].

Narrow band imaging

NBI (Figure 1) is one of the most widely used and ex-

Table 1 Endoscopic innovations to improve the adenoma detection during colonoscopy

Technique	Colonoscopy technology	Diagnostic yield	Clinical applicability
High definition	High definition monitor with more images per second and high resolution	Marginal increase in number of polyps and adenomas, mostly small, flat, right-sided lesions. approximation 3.5% increase in ADR	High quality images with reduced artifacts and more natural appearance
Narrow band imaging (NBI)	Narrow band filters increase blue (415 nm) and green (540 nm) wavelengths and enhance the visualization of mucosal blood vessels	Small increase in flat and small serrated lesions, but higher detection rates when combined with high definition	Possibly improving the detection of subtle lesions, but insufficient brightness and dark appearing bile and stool prohibit optimal pan-colonic use
Fujinon intelligent color enhancement	Computed spectral estimation technology enhances the visibility of mucosal and vascular details by narrowing the bandwidth of light	Very few randomized studies but polyp and adenoma detection seems similar compared to white light colonoscopy	Like with NBI, images are to dark to advice routine pan-colonic use
Autofluorescence imaging (AFI)	Tissue is exposured to light of short wavelength, which leads to the excitation of endogenous substances and the emission of autofluorescent light	AFI has lower adenoma miss rates (absolute difference of approximation 20%) when compared to white light colonoscopy, especially for flat and depressed lesions	Not advised for routine practice in colonoscopy due to low resolution images, few images per second and artifacts due to residual fecal fluids
Water-immersion colonoscopy	Infusion of water, combinated with air-insufflation, during insertion of the colonoscope. Water and remaining fecal content are removed during withdrawal	No difference in ADR between water-immersion and air-insufflated colonoscopy	Reduces pain scores, need for sedation and general intolerability, but only studied in highly experienced hands
Water-exchange colonoscopy	Water containing residual feces is removed and "exchanged" for clean water during insertion in lieu of air-insufflation	ADR is reported to be approximation 10% higher with water-exchange colonoscopy compared to standard air-insufflated colonoscopy	Provides extra cleansing of the mucosa but is more time consuming and is thus far only studied in highly experienced hands
Cap-assisted colonoscopy	Can be used to depress colonic folds to improve the visualization of proximal aspects of these folds	Contradicting results with approximation 10% higher detection rates for small polyps and adenomas in some studies, but no beneficial results in others	Easy to use, can assist during mucosectomies and facilitates introduction of the colonoscope, but probably has a limited effect on diagnostic yield
Retroflexion	Withdrawal in retroflexion is possible in the proximal colon due to the large diameter of this segment and may improve the visualization of the proximal aspects of folds	No additional diagnostic yield in the proximal colon and questionable in the rectum	Routine withdrawal in retroflexion is not recommended but may facilitate the removal of large sessile polyps
Third-eye retroscope	The retroscope is retroflexed 180 degrees after being advanced through the working channel and enhances the visualization behind folds	Limited number of studies, but polyp and adenoma detection are reported to be 15%-25% higher compared to standard colonoscopy	Increases diagnostic yield, but reduces suctioning capacity when in position and needs to be removed from working channel in case of polypectomy
Full spectrum endoscopy (FUSE)	Three imagers positioned at the front and both sides of the tip provide a 330 degrees view, which improve the visualization of the internal lining of flexures and proximal aspects of folds	One randomized tandem study thus far, which showed considerably lower miss rates for polyps (9.7% *vs* 43.%) and adenomas (7.5% *vs* 40.8%) compared to standard colonoscopy	Provides a comprehensive view while maintaining technical capabilities of standard colonoscopes. Requires little training

ADR: Adenoma detection rate.

tensively studied image enhancement technologies and is aimed to improve adenoma detection and differentiation. Narrow band filters placed behind the light source eliminate red light and increase the contribution of blue (415 nm) and green (540 nm) wavelengths. The 415 nm light enhances the visualization of superficial mucosal capillaries while the 540 nm light increases the visibility of submucosal and deeper mucosal vessels.

Studies investigating the additional yield of pan-colonic NBI are somewhat conflicting. A meta-analysis including six randomized trials with a total of 2284 patients[23] reported no significant differences between high definition NBI and high definition white light colonoscopy for the detection of total, flat and < 10 mm adenomas or polyps. Furthermore, no differences in adenoma or polyp miss rates were observed between both techniques. These findings were recently confirmed in a large randomized study by Chung *et al*[25]. In contrast, studies in which high definition NBI was compared to standard definition colonoscopy have shown differences in detection and miss rates of polyps and adenomas[18,26,27]. In a randomized back-to-back study by Gross *et al*[27] comparing high definition NBI and standard definition colonoscopy, significant lower miss rates for polyps (31% *vs* 57%, $P = 0.005$) and adenomas (27% *vs* 49%, $P = 0.036$) were observed. However, the same group reported similar results in a retrospective study comparing high definition white light to standard definition white light colonoscopy, which suggests that the additional yield obtained with high definition NBI may be related to the high definition component and not to the use of NBI[20]. This is further supported by a study of Rastogi *et al*[18] in which more adenomas per subjects were found with high definition NBI (1.13) compared to standard

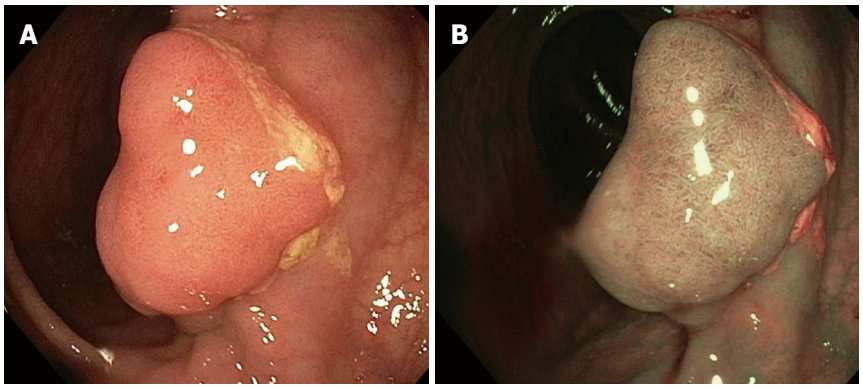

Figure 1 View of adenomatous polyp with white light and narrow band imaging. A: Adenoma view with white light; B: Adenoma view with narrow band imaging.

definition white light (1.13 *vs* 0.69, $P = 0.01$) but not to high definition white light colonoscopy (1.13 *vs* 1.12, $P > 0.05$). In the latter study, high definition NBI detected significantly more flat and right sided lesions compared to standard definition colonoscopy but a similar number compared to high definition colonoscopy. A back-to-back study including patients with hyperplastic polyposis syndrome also reported a lower polyp miss rate, in particular for flat polyps and sessile serrated adenomas, when high resolution NBI colonoscopy was compared to white light colonoscopy[28]. Two randomized studies that compared high definition NBI with high definition white light colonoscopy[29,30] reported no difference in adenoma detection, but high definition NBI yielded more flat adenomas[29,30] and hyperplastic polyps[29].

In summary, polyp and adenoma detection seem to be higher with high definition NBI compared to standard definition white light colonoscopy, but the additional value of high definition NBI over high definition white light colonoscopy may be limited to the detection of subtle lesions such as small serrated lesions and flat adenomas. It has been suggested that the limited value of high definition NBI over high definition white light colonoscopy may be related to the potential learning effect that is induced by NBI during colonoscopy, *i.e.,* the introduction of NBI may have improved the recognition of polyps and adenomas with white light colonoscopy[23]. In this regard, it is of interest that East *et al*[31-33] showed that the improvement in adenoma detection rate by high definition NBI colonoscopy over high definition white light colonoscopy declined from 61% in the first 52 patients to 45% and only 8% in a second and third group of 91 and 214 patients. A similar effect was observed in a study by Adler *et al*[34] with consecutive groups of 100 patients undergoing white light colonoscopy; the ADR of 8% in the first group increased to 26% in the last group of patients, compared to an ADR of 25% with NBI which remained unchanged during the course of the study.

FICE

FICE is a computed spectral estimation technology that enhances the visibility of mucosal and vascular details by narrowing the bandwidth of light. FICE enables the endoscopist to choose between different wavelengths for optimal examination of the colon mucosa[24]. Only a limited number of studies have evaluated FICE colonoscopy for its proposed increased capability to detect of adenomas and polyps. In the reported randomized back-to-back studies that compared FICE with white light colonoscopy[25,35] or NBI[36] no significant benefit of FICE was demonstrated. Furthermore, in an earlier randomized study[37] the ADR and mean number of adenomas were similar with FICE compared to targeted indigocarmine chromoendoscopy.

AFI

Real-time pseudo-color images produced with AFI are created by a rotating filter producing a short wavelength light. The exposure of tissue to this specific light leads to the excitation of some endogenous substances and subsequently the emission of fluorescent light. The autofluorescent image produced with AFI is created by a green filter, which exposes the tissue to the remaining blue and red light. The reflected blue light is blocked by a second filter while the reflected red light and the emitted green autofluorescence from the tissue are used to obtain a pseudo-color image[22,38]. AFI colonoscopy colors neoplastic lesions red-purple while non-neoplastic mucosa appears green.

Three back-to-back studies reported lower adenoma miss rates with AFI colonoscopy compared to white light colonoscopy with an absolute difference of approximately 20%[39-41]. In one of these studies[39], the location, size, macroscopic appearance and histopathology of the lesions detected with AFI and white light colonoscopy were not different, but the lesions that were histologically graded as dysplastic were less frequently missed with AFI (30% *vs* 49%, $P = 0.01$). Another study by Moriichi *et al*[40] compared AFI with high resolution white light colonoscopy and reported a higher ADR (26.1% *vs* 18.2%, $P < 0.05$) and more specifically a higher detection rate of flat and depressed adenomas (9.1% *vs* 3.4%, $P < 0.05$). In the same study, an increased ADR with AFI was only observed when used by less experienced endoscopists. One study investigated the diagnostic

yield of high resolution colonoscopy using Endoscopic Trimodal Imaging technology[42]. These colonoscopes have both AFI and NBI technology incorporated in the endoscope. The high resolution and AFI technology in these colonoscopes can be used to detect lesions ("red flag"), whereas NBI can be used to differentiate between different types of lesions. The study was performed in six non-academic centers and showed no differences in ADR or adenoma miss rate compared to standard white light colonoscopy.

In summary, the effect of pan-colonic virtual chromoendoscopy on adenoma and polyp detection seems limited and virtual chromoendoscopy probably only has a minor benefit on the detection of small and flat lesions. These somewhat disappointing results are most likely due to technical issues inherent to virtual chromoendoscopy, in that the brightness of the virtual image with high-definition technology remains insufficient to allow optimal visualization of the colonic mucosa in a large diameter colon lumen. In addition, a good inspection of the colon mucosa with virtual chromoendoscopy is only possible in a colon that is really optimally prepared because remaining bile fluid and stool appear red and dark in virtual images, hindering an optimal view of the mucosa[43]. In our opinion, virtual chromoendoscopy is most optimally used as an add-on technology to differentiate between neoplastic and non-neoplastic lesions. This could allow a "resect-and-discard" or "leave-in-situ" approach to reduce the risk of complications and costs associated with unnecessary removal of polyps. However, accuracy rates should exceed well above 90% to consider such an approach. In experienced hands, high accuracy rates for a "resect-and-discard" policy have been reported for NBI[44-46], FICE[47,48] and AFI[49], ranging between 85%-92% when used with high magnification, but these rates are lower when used by non-experts[42,44,50]. Good training may improve the detection and differentiation of lesions, but before the routine use of pan-colonic virtual chromoendoscopy can be justified, new generation devices with higher light intensity are required.

WATER-INFUSION TECHNIQUES

Colonoscopy techniques combining or replacing air-insufflation with water infusion were initially designed to facilitate cecal intubation, reduce colonic spasms, lower patient discomfort and need for sedation[51,52]. The infusion of water during the insertion of the colonoscope causes the colon to distend and can be combined with air-insufflation (water-immersion method) or be performed without air-insufflation (water-exchange method)[53,54]. Similarly to standard air-insufflated colonoscopy, air is also insufflated during withdrawal of the colonoscope irrespective of the type of water infusion technique used. The water-immersion technique allows the water to flow in the direction of the lumen which may aid in finding the correct direction for intubation. The infused water and remaining fecal contents are mainly removed during withdrawal of the colonoscope.

This method has been shown to reduce pain scores[55-61], need for sedation[55,59,62] and general intolerability[55,59,60] in most studies, but concerns have been raised about an impaired ability to detect lesions due to contaminated water impairing visibility. A recent systematic review[53] in which the results of six studies were combined, reported no differences in ADR between water-immersion and air-insufflated colonoscopy. In contrast, the recently developed water-exchange method was reported to increase ADR compared to air-insufflation colonoscopy in the first observational study (water-exchange 36.5% *vs* air 25.8%, $P = 0.18$)[63], in a subsequent retrospective cohort study (water-exchange 34.9% *vs* air 26.9%, $P = 0.003$)[64] and in a head-to-head comparison study (water-exchange 57.1% *vs* air 46.1%, $P = 0.04$)[65]. In two randomized controlled trials[62,66], ADR was higher with the water-exchange method but this difference was not statistically significant. The water-exchange method is a technique in which water containing residual feces is removed and "exchanged" for clean water in lieu of air-insufflation. The exchange of large volumes of water during the insertion of the colonoscope results in additional cleansing of the mucosa, which has been proposed to improve the detection of adenomas[53]. An alternative hypothesis is that the improved cleansing during colonoscope insertion allows more time for inspection during withdrawal since less time needs to be spent on colonic cleansing. Nonetheless, several attempts have been made to improve the efficacy of water-exchange colonoscopy. In a group of 50 consecutive US veterans undergoing water-exchange colonoscopy, indigocarmine was added to the infused water (concentration 0.008%)[67]. The ADR was significantly higher in the indigocarmine group in comparison with a historical cohort of patients who had undergone standard water-exchange colonoscopy (62% *vs* 40%, $P < 0.05$) or air-insufflation colonoscopy (62% *vs* 36%, $P < 0.05$). In a pilot study by Yen *et al*[68], the water-exchange method was combined with cap assisted colonoscopy in 50 consecutive patients. The results were compared to a control group of 101 consecutive patients undergoing air-insufflation colonoscopy. It was demonstrated that the mean number of adenomas was higher with the water-exchange cap assisted colonoscopy method compared to air-insufflated colonoscopy (3.08 *vs* 1.50, $P = 0.002$), although the ADR was not statistically significantly higher (70.0% *vs* 59.4%, $P = 0.22$).

Although water-exchange colonoscopy improves the detection of adenomas, the benefit of water-infusion colonoscopy methods seems particularly be due to improving patient comfort. In addition, the majority of studies published so far were performed by endoscopists that were highly experienced with water-infusion colonoscopy. This raises the question whether the same results can be achieved when performed by less experienced endoscopists. Especially when considering the

prolonged insertion time due to the time consuming suction and exchange of water, it remains to be further elucidated whether water-exchange colonoscopy will indeed be one of the preferred techniques in daily clinical colonoscopy practice.

CAP-ASSISTED COLONOSCOPY

Transparent caps attached to the distal tip of the colonoscope were first designed to assist during endoscopic mucosal resection but they have also been suggested to be of help in depressing colonic folds to improve visualization of their proximal aspects. A potential disadvantage of cap-assisted colonoscopy is that fecal debris may accumulate in the cap, requiring removal by water irrigation and drainage through the side holes present in some cap models. Several studies have reported reduced cecal intubation times[69-71] and improved cecal intubation rates for trainees using cap-assisted colonoscopy[70]. The same accounted for procedures in patients in whom cecal intubation initially failed with standard colonoscopy[72,73]. Randomized controlled trials that evaluated the additional diagnostic yield of cap-assisted colonoscopy were mostly performed in Asian countries and have in general shown mixed results[74].

In a study by Kondo *et al*[70], 684 subjects were randomized to colonoscopy with a 4-mm transparent cap or a 2-mm rubber cap or to colonoscopy without a cap. PDR for colonoscopies with the transparent cap, rubber cap and no cap were 49.3%, 44.7% and 39.1%, respectively, with only the difference between the transparent 4-mm cap and no cap being statistically significant. In a recent study reporting on 2502 procedures performed by trainees[75], a statistically significant higher overall PDR was found for cap-assisted colonoscopy compared to standard colonoscopy (47.0% *vs* 42.6%). Subgroup analyses showed that this difference was particularly due to an improved detection of small (≤ 5 mm) polyps. In a randomized controlled trial by Rastogi *et al*[71], ADR was 13% higher with cap-assisted colonoscopy compared to standard colonoscopy, but similarly to the previous study, this was only observed for small (≤ 5 mm) adenomas. Horiuchi *et al*[76] studied a retractable transparent device that can be extended up to a maximum length of 7 mm by injection of air. The mean number of adenomas detected was statistically significantly higher with the retractable extension device compared to standard colonoscopy (0.48 *vs* 0.36, $P = 0.04$) while the ADR was comparable between both groups. In contrast, in the single largest randomized trial[73] published thus far (1000 patients included), a lower ADR (30.5% *vs* 37.5%) and mean number of adenomas per subject was reported with cap-assisted colonoscopy compared to standard colonoscopy. Furthermore, three later studies, including the largest published multicenter trial thus far, reported no higher overall[69,77,78] and small polyp[69,77,78] detection rates with cap-assisted colonoscopy.

Taken together, cap-assisted colonoscopy may be of benefit in reducing cecal intubation time, but has limited or no benefit on polyp detection, which is confirmed by the results of a recent meta-analysis including 16 randomized controlled trials[74]. In this study, a marginally higher proportion of subjects with polyps was found with cap-assisted colonoscopy (RR = 1.08, 95%CI: 1.00-1.17) while no statistically significant difference in ADR was found. Of note, subgroup analysis showed that both expert and trainee endoscopists had reduced cecal intubation times and improved polyp detection rate, highlighting that it is unlikely that especially trainees should benefit from cap-assisted colonoscopy.

RETROFLEXION

Retroflexion is commonly used in the rectum for the inspection of the dentate line, though the additional diagnostic yield is questionable[79]. Due to its relatively large diameter, the retroflexion technique has also been suggested to be useful in the proximal colon to improve the visualization on the proximal aspects of folds and to facilitate the removal of proximally located large sessile polyps. This was shown in a retrospective observational study in 59 patients[80].

Harrison *et al*[81] performed a randomized study in 100 patients who underwent standard forward colonoscopy from the cecum to the splenic flexure with removal of polyps. The cecum was then reintubated and patients were randomized to undergo a second exam of the proximal colon in retroflexion or in forward view. Retroflexion was successfully performed in the ascending and transverse colon in almost all patients. No statistically significant differences were observed between forward view and retroflexion with regard to the detection of additional polyps and adenomas. A more recent observational study in a cohort of 1000 consecutive patients reported an adenoma miss rate of 9.8% in patients first undergoing careful inspection of the proximal colon in forward view and a second inspection in retroflexion[82]. Although this was an observational study, the adenoma miss rate was thought to be comparable to that expected when a second inspection would have been done with forward viewing colonoscopy.

Based on the relatively limited number of studies which demonstrated no clear extra additional polyps being detected, in combination with a possibly increased risk of perforation when withdrawing the colonoscope in retroflexion, we currently do not recommend this technique in routine colonoscopy practice.

THIRD-EYE RETROSCOPE

A device specifically designed to enhance the visualization behind the proximal aspects of colonic folds is the Third-Eye Retroscope® (Avantis Medical Systems, Inc) (Figure 2). This device consists of a video processor, a single-use polarizing filter cap for the colonoscope light source, and a 3.5 mm flexible single-use catheter with a

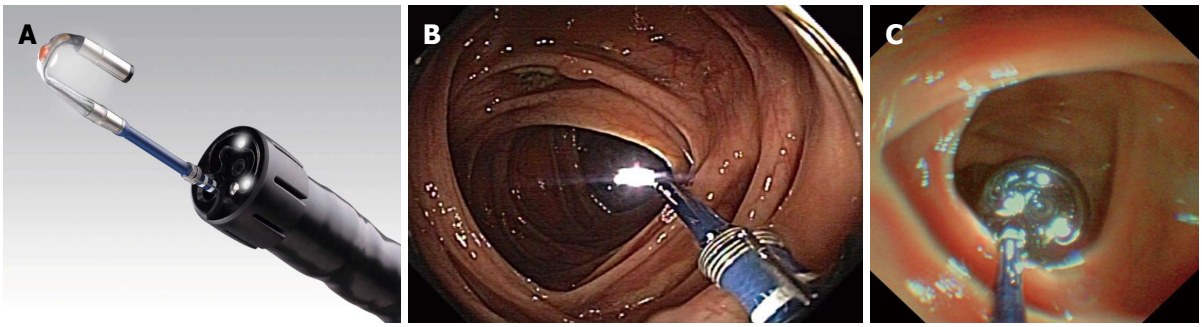

Figure 2 Colonoscopy with the Third-Eye Retroscope. A: Detailed view of the Third-Eye Retroscope; B, C: Colon view with the Third-Eye Retroscope.

camera and diode light source at the tip. The retroscope is retroflexed 180 degrees after being advanced through the working channel of the colonoscope and provides a 135 degrees retrograde view of the colon. In simulated colonoscopies using CT-colonography software, it was shown that the Third-Eye Retroscope improves the visualization of the colonic surface area from 87% with standard 140 degrees view colonoscopes to 99%[13].

The efficacy of the Third-Eye Retroscope was initially studied in three colon models with simulated polyps[83]. Standard colonoscopy detected 12% of the polyps located on the proximal aspects of folds, while 81% of these polyps were detected with the Third-Eye Retroscope. The first pilot study[84] in 24 patients resulted in an 11.8% increase in diagnostic yield, with 34 polyps detected in the antegrade view and 4 additional polyps in the retrograde view. In two non-randomized studies[85,86], the additional diagnostic yield of the Third-Eye Retroscope was investigated by evaluating whether polyps detected with the Third-Eye Retroscope could also been seen with the antegrade view of the colonoscope alone. In the first study, 182 polyps in 298 subjects were found with the antegrade view and 27 additional polyps were found with the Third-Eye Retroscope, resulting in a 14.8% increase in polyp detection and a 16.0% increase in adenoma detection[85]. The second study reported a similar result with a 13.2% increase in polyp detection and a 11.0% increase in adenoma detection[86]. Until now, one randomized back-to-back study has been performed, the TERRACE study[87]. In this multicenter study including 349 patients, a net additional detection rate with the Third-Eye Retroscope of 29.8% for polyps and 23.2% for adenomas was reported. This study was criticized by the fact that the mean withdrawal time was almost two min longer with the Third-Eye Retroscope compared to standard colonoscopy as this may have resulted in some bias in this study. In a post-hoc analysis of the TERRACE study[88], withdrawal time was not significantly associated with the risk of missing adenomas. Interestingly, the Third-Eye Retroscope was shown to be particularly beneficial in patients undergoing colonoscopy for surveillance or diagnostic work-up and not in those undergoing screening colonoscopy.

Studies that investigated the Third-Eye Retroscope have shown a significant additional diagnostic yield when using this technique, but there are some limitations inherent to this device. First, thorough suctioning of colonic debris must be done during insertion of the colonoscope due to a 50% reduced suctioning capacity when the retroscope is in position. A second disadvantage is that the Third-Eye Retroscope needs to be removed from the working channel in case an accessory device is required, such as a biopsy forces or a polypectomy snare, which is bothersome and increases the procedural time.

FULL SPECTRUM ENDOSCOPY

The recently developed Full Spectrum Endoscopy™ (FUSE; EndoChoice®, Alpharetta, Georgia, United States) (Figure 3) colonoscope allows a high resolution 330 degrees "full spectrum" viewing of the colonic lumen while maintaining the standard colonoscope technical features and capabilities of a standard 140 and 170 degrees colonoscope. The FUSE system consists of a main control unit and a video colonoscope with three imagers and LED groups located at the front and both sides of the flexible tip. The video images transmitted from the three cameras on the left-side, front and right-side of the colonoscope are displayed on three contiguous monitors corresponding with each individual camera. The two additional side cameras incorporated in the FUSE colonoscope provide a better and comprehensive view of the total colonic lumen. The frequently encountered blind spots, such as the internal lining of flexures and proximal aspects of folds, should be easily visualized with this system.

The first published study was performed with an anatomical model of the colon with simulated polyps in a non-randomized setting[89]. Thirty-seven endoscopists performed colonoscopy by using the forward-viewing camera only (160 degrees), followed by a colonoscopy with all three cameras, which increases the field of view to 330 degrees. In total, 85.7% of the polyps were detected with the three cameras compared to 52.9% with only forward-viewing colonoscopy ($P < 0.001$). Particularly polyps that were "hidden" behind flexures and folds were more frequently detected with FUSE colonoscopy than with forward-viewing colonoscopy (81.9% *vs* 31.9%). An additional pilot study including 50 patients showed that FUSE colonoscopy was indeed safe and

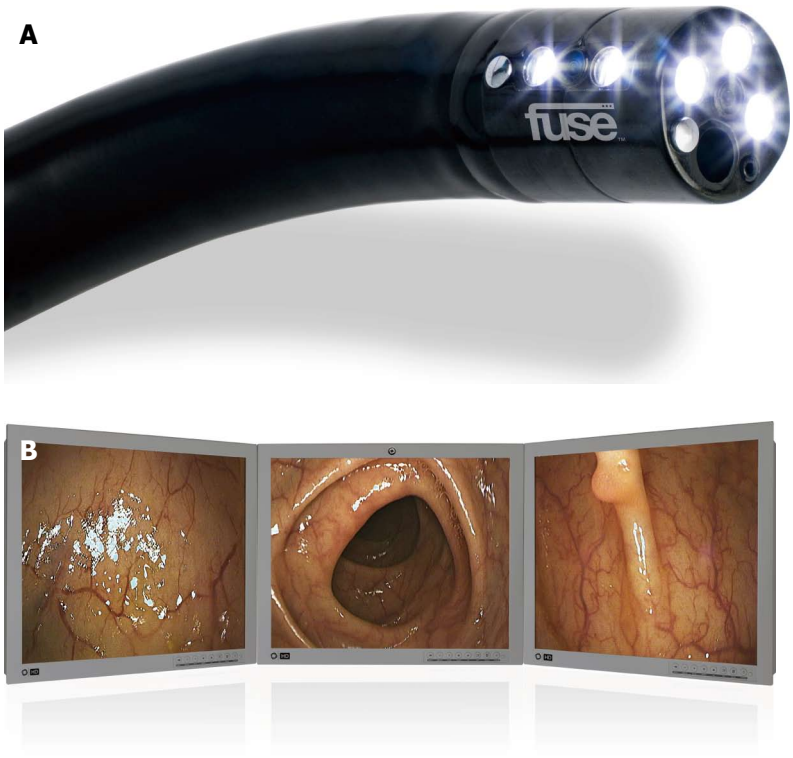

Figure 3 Colonoscopy with the Full Spectrum Endoscopy colonoscope. A: Detailed view of the Full Spectrum Endoscopy colonoscope; B: Colon view with the Full Spectrum Endoscopy colonoscope.

feasible with a 100% cecal intubation rate and a mean cecal intubation time of 3.1 ± 1.5 min. Preliminary results of a randomized, multicenter, back-to-back study presented at the Digestive Disease Week 2013 are promising. Same-day colonoscopies with FUSE and standard colonoscopes were performed in 185 randomized subjects. In 88 subjects undergoing standard colonoscopy first, 50 polyps including 28 adenomas were detected while FUSE yielded 39 additional polyps including 20 adenomas, corresponding with an increase in polyps and adenomas detection of 78.0% and 71.4%, respectively. In 97 subjects undergoing FUSE first, 102 polyps including 61 adenomas were detected while standard colonoscopy yielded 11 additional polyps including 5 adenomas, corresponding with an increase in polyps and adenomas of 10.8% and 8.2%, respectively (FUSE *vs* standard, $P < 0.01$). The adenoma miss rate with FUSE was found to be considerably lower than with standard colonoscopy (7.5% *vs* 40.8%, $P < 0.0001$). However, the median withdrawal time was approximately half a minute longer with FUSE colonoscopy (5.6 min *vs* 6.2 min, $P < 0.01$) and may have caused some bias in the results. More studies will be required before definitive conclusions can be made, but the first results definitely show that FUSE colonoscopy may be an important advancement to improve adenoma detection.

CONCLUSION

A considerable proportion of polyps and adenomas are missed during colonoscopy due to poor visualization behind folds and the inner curves of flexures, and the presence of flat lesions that are known to be difficult to detect. Based on the findings of back-to-back studies with standard colonoscopes, adenoma and polyp miss rates are estimated to be approximately 20% to 25%. However, some recent studies that evaluated new endoscopic technologies have reported even higher miss rates (up to 40%) with standard colonoscopy than previously reported, which suggests that the miss rates with standard colonoscopy may have been previously underestimated.

The introduction of high-definition technology has considerably improved the quality of images during colonoscopy and is likely to stay the standard in the field of endoscopy. Visual image enhancement technologies such as NBI, FICE and AFI have possibly resulted in an increased recognition of flat and small lesions, but the absolute increase in terms of numbers of adenoma is probably limited. Besides, the quality of the images produced with virtual chromoendoscopy technologies requires further improvement before the general application of such technologies can be fully recommended. Cap-assisted colonoscopy and water-exchange colonoscopy were originally designed to facilitate cecal intubation and increase patient comfort, but studies have generally shown a marginal or no benefit at all on polyp and adenoma detection. Furthermore, the applicability of water-infusion methods has only been studied in highly experienced hands and is more time consuming compared to standard colonoscopy. Retroflexion is com-

monly used in the rectum for the inspection of the dentate line, but its use in the proximal colon has not clearly been demonstrated to improve ADR and may be associated with an increased risk of perforation. Studies evaluating colonoscopy with the Third-Eye Retroscope have shown considerable lower miss rates compared to standard colonoscopy, but this device is inconvenient in case of polypectomy, it impacts suction capabilities and it adds to total colonoscopy time. The recently introduced FUSE colonoscope maintains the technical capabilities of standard colonoscopes and provides a much wider view of 330 degrees compared to 170 degrees with standard colonoscopes. A recent randomized back-to-back study using FUSE colonoscopy has shown remarkable lower adenoma miss rates with this technique. Although the results look promising, more studies investigating the diagnostic yield and the use of three monitors are needed before this device can be recommended for routine practice.

Hence, the majority of the endoscopic innovations that have been introduced in the past few years have only shown little additional diagnostic yield, are more time consuming or are not practical in use. In order to increase the efficacy of screening and surveillance colonoscopies, colonoscopy techniques will be needed that provide an optimal view of the whole colonic mucosa while maintaining optimal washing, suction and therapeutic capabilities and without increasing the procedural time or impairing patients comfort. In this perspective, a combination of high-definition and improved virtual enhancement technologies incorporated in ultra-wide colonoscopes may be the most obvious way to enhance the diagnostic yield of colonoscopy in the next few years.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (13): Gastrointestinal endoscopy

Biodegradable stents in gastrointestinal endoscopy

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Abstract

Biodegradable stents (BDSs) are an attractive option to avoid ongoing dilation or surgery in patients with benign stenoses of the small and large intestines. The experience with the currently the only BDS for endoscopic placement, made of Poly-dioxanone, have shown promising results. However some aspects should be improved as are the fact that BDSs lose their radial force over time due to the degradable material, and that can cause stent-induced mucosal or parenchymal injury. This complication rate and modest clinical efficacy has to be carefully considered in individual patients prior to placement of BDSs. Otherwise, the price of these stents therefore it is nowadays an important limitation.

Key words: Biodegradable stents; Strictures; Endoscopy; Endoscopic placement; Stenoses

Core tip: The experience with the currently the only biodegradable stents (BDSs) for endoscopic placement, made of poly-dioxanone, have shown promising results. However some aspects should be improved as are the fact that BDSs lose their radial force over time due to the degradable material, and that can cause stent-induced mucosal or parenchymal injury. This complication rate and modest clinical efficacy has to be carefully considered in individual patients prior to placement of BDSs. Otherwise, the price of these stents therefore it is nowadays an important limitation.

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INTRODUCTION

Metal and plastic stents are an effective treatment to manage both benign and malignant strictures throughout the gastrointestinal (GI) tract; however, the use of these stents is associated with several common problems including migration, tissue ingrowths and repetitive endoscopic procedures. During the last two decades, significant advances have been made in the development of biocompatible and biodegradable materials for medical applications, and to overcome those shortcomings, stents made of biodegradable materials have been developed. The cardiovascular stent market is the dominant driving force for research and development of biodegradable stents (BDSs)[1]. In the GI tract much less is currently known about the clinical utility and the experience with these stents.

BIOMATERIALS

Biomaterial is a non-living material used in a medical

device and designed to interact with biological systems. Biomaterial can be (1) inert, do not trigger any reaction in the host; (2) bioactive, ensure a more stable performance in a long time or for the period you want; or (3) biodegradable, it can be chemically degraded or decomposed by natural effectors as bacteria. The mean features of these materials for medical applications are absence of carcinogenicity, immunogenicity, teratogenicity and toxicity.

More used biomaterials are magnesium alloys based and synthetic polymers: poly-lactic acid (PLA), polyglycolic acid (PGA), poly-caprolactone (PCL), polydioxanone (PDX) and poly-lactide-co-glycolide.

Main advantages of magnesium alloys based are high biocompatibility and property to be dissolved into human body during the degradation process; however, due to this high corrosion rate, degradation occurs before the end of healing process.

Polymers degrade slower than magnesium alloys. Most polymers used in medical devices allow the spread of water within molecular structure and can therefore result in processes hydrolysis. The ideal polymer should be: (1) sufficiently strong until surrounding tissue has healed; (2) does not invoke inflammatory or toxic response; (3) to be metabolized in the body after fulfilling its purpose; (4) leaving no trace to be easily processable into the final product form; (5) must demonstrates acceptable shelf life; and (6) to be easily sterilized. With these characteristics; the main advantages of synthetic polymers are: (1) good biocompatibility; (2) possibility of changing in composition and in physical-mechanical properties; (3) low coefficients of friction; (4) easy processing and workability; (5) ability to change surface chemically and physically; and (6) ability to immobilize cells or biomolecules within them or on the surface (Drug Eluting Stent). Biodegradable polymers used for drug release represent the next technological modification and preliminary results are favorable in vascular system and clinical efficacy as first-generation drug eluting stent, but gastrointestinal application has not been reported yet[2].

According to these features, BDS can be made of different synthetic polymers (PLA, PGA) or their copolymers (PDX). Their degradation is hydrolytic and the speed of biodegradation is dependent not only on the size and structure, but also influenced by temperature, pH and type of body tissue/fluid[3,4].

CLINICAL EXPERIENCE

The first report was published in 1993[5] in an experimental model of urethral stenosis with rabbits treated with a stent made of PLA. In gastrointestinal endoscopy, BDSs made of PLA were developed by Goldin *et al*[6], who reported their experience with five patients with benign esophageal strictures. These authors described that this prototype did not maintain significantly radial force over a 3-wk period, and disintegrated 6 wk later and obstructed the esophageal lumen, data that was confirmed by another group[7].

BDSs made of PDX have improved the results because stent integrity and radial force can be maintained for 6-8 wk after implantation[8]. In this pilot study with three patients with benign tight small and large intestinal stenoses, the authors confirmed that stent degradation and fragmentation occurs 11-12 wk after its insertion; otherwise, the speed of degradation is pH-dependent (faster in lower pH).

The prolonged dilatory effect before stent absorption and the progressive stent degradation could represent a more favorable solution for patients with benign strictures refractory to standard dilation therapy compared with self-expandable metal and plastic stents. These newly stents allow for constant radial dilation, similar to date achieved by a metallic stent, but with the advantage that they do not have to be removed. To date, PDX-BDSs could be an alternative for benign refractory strictures in the GI tract.

Esophagus

Placement of BDS is an emerging and promising treatment alternative for benign esophageal strictures and achalasia. These strictures are often caused by esophageal reflux, the ingestion of caustic substances, esophageal surgery and radiation therapy[9]. Endoscopic dilation using bougies or balloons has been established as a standard therapy, and it is associated with an immediate 80%-90% success rate of relieving dysphagia[10]. However, 30%-60% of benign strictures will recur during long-term follow-up; thus, an alternative treatment strategy should be considered[11].

BDSs have recently been developed. Initially, these stents were made of PLA, with a configuration and mechanical radial force similar to those commercially available esophageal stents[12]. Saito *et al*[13,14] reported results from two series of patients who received this type of PLA esophageal stents, but the majority (77%) of stents had migrated out of the esophagus within 10-21 d of insertion, although clinical success was observed in all cases within the follow-up period of 7 mo to 2 years.

Another novel stent (Ella esophageal stent) composed of the biodegradable polymer PDX has become to be used from 2007 (and currently the only). This stent is assembled onto a 9.4-mm (28 F) delivery system, and comes in several sizes, with stent body diameters ranging from 18 to 25 mm and fully deployed lengths of 60-135 mm. Integrity and radial force are completely maintained for approximately 6 wk following implantation. From 7-9 wk is 2/3 of the initial, after the 9 wk is 1/3, and the average time to complete degradation of the stent is reported to be 11-12 wk[8]. Acid-suppressing therapy is recommended, because more rapid degradation occurs with acid exposure.

Published experience has been reported with the SX-ELLA-BD stent for treatment of refractory benign esophageal strictures (RBES)[9,15-29] as well as achalasia[30] (Table 1). Technical success, clinical responses and out-

Table 1 Cases series evaluating the outcomes of biodegradable stents with polydioxanone for refractory benign esophageal strictures

Ref.	Year	Type of study	*n*	Follow-up (mo)	Technical success	Complications	Clinical success
Dhar *et al*[23]	2009	CS	4	4	100%	Chest pain, 25%	50%
Vandenplas *et al*[15]	2009	CR	1	4	100%	0%	100%
Orive-Calzada *et al*[16]	2009	CR	1	3	100%	T. hyperplasia, 100%	0%
Stivaros *et al*[27]	2010	CS	2	7	100%	Chest pain, 50% Stent migration, 50%	50%
Bychkova *et al*[24]	2009	CR	1	4	100%	0%	100%
Hair *et al*[30]	2010	CR	1	8	100%	T. hyperplasia, 100%	0%
van Hooft *et al*[17]	2011	CS	10	6	100%	Chest pain, 20%	60%
Repici *et al*[29]	2010	CS	21	13.2	100%	Chest pain, 14% Hemorrhage, 5% Stent migration, 9%	45%
Nogales Rincon *et al*[21]	2011	CR	1	2	100%	Stent collapse, 100%	0%
Martín Cano *et al*[25]	2012	CS	3	4	100%	0%	100%
Griffiths *et al*[28]	2012	CS	7	4	96%	0%	76%
Canena *et al*[9]	2012	RCT	10	12	100%	Chest pain, 10% Hemorrhage, 10% Stent migration, 20 T. hyperplasia, 30%	30%
Fischer *et al*[19]	2012	CS	2	12	100%	T. hyperplasia, 50%	50%
Dumoulin *et al*[20]	2012	CR	1	18	100%	T. hyperplasia, 100%	0%
Hirdes *et al*[22]	2012	CS	28	10	100%	Chest pain, 22% Hemorrhage, 8%	25%
Basha *et al*[18]	2013	CR	1	4	100%	0%	0%
Karakan *et al*[26]	2013	CS	7	15	100%	T. hyperplasia, 57%	43%

CR: Case report; CS: Case series; RCT: Randomized clinical trial; T. hyperplasia: Tissue hyperplasia.

comes were varied. Stent implantation is not a problem, but clinical success ranged from 0 to 100, with a mean of 39.4%. The largest long-term follow-up series of BDSs placement in 28 patients with RBES were reported by Hirdes *et al*[22], with a clinical success rate after the first BDS placement of 25%.

To avoid complications of partially covered/uncovered stents, temporary placement of 3 different types of expandable stents have been used for the treatment of RBES: self-expanding plastic stents (SEPSs), BDSs and fully covered self-expandable metal stents (FC-SEMSs). To date there is only one published study that compares the efficacy of these three types of stent for the treatment of RBES[9]. These authors showed that temporary placement of BDSs or FC-SEMSs have similar utility in the treatment of RBES, with a long-term dysphagia-free period in 30% and 40% of patients, respectively. The use of SEPSs were associated with the worst clinical success rate (10%) as well as with a higher number of migrations and reinterventions. A long stricture was the only significant fact associated with a higher recurrence rate after stent placement. Migration rate was higher with FC-SEMSs (30%) than with BDSs (20%). The implementation of balloon dilatation of the BDS stent after deployment did decrease migration rated compared to before balloon dilation.

Thoracic pain is the most frequent complication reported in the literature. Hyperplasic tissue reaction occurs in conjunction with stent degradation and the severity of the tissue response and the time to complete degradation, both important factors when considering patients for placement of a BDS, are still well not understood. Cases of severe tissue hyperplasia resulting in recurrent dysphagia have been described[16,19,20,26,30]. To alleviate symptoms in this type of stent, endoscopic balloon dilation[26,30], or argon plasma coagulation have been reported[20], but there are no recommendations to guide what endoscopic approach is best. Other potential complications of BDS that have been addressed are collapse of the biodegradable stent mesh inside the esophageal lumen[21] and tracheoesophageal fistula[31]. Finally, fully covered BDSs could be useful in the treatment of esophageal perforations and anastomotic leaks[32].

Small intestine and colon

BDSs are a promising therapeutic option for benign intestinal and colonic strictures. Strictures following colorectal surgery are the most frequent, and occur in 1.5%-8% of patients[33]. Published information is limited to patients with anastomotic colorectal strictures following resection for colon cancer[34-37], postsurgical colonic fistulas[35], and stricturing Crohn's disease[38,39] (Table 2). Intestinal insertion is technically possible and relatively simple, except for proximal stenoses and in patients with considerable deformity and angulations. The standard delivery system of PDX-BDS has an active length of 75 cm that limitates proximal stent insertion, and in most cases it may not be possible to place the stent at more than 30 cm from the anus[35]. Technical difficulties for more proximal stenoses are pre-empted due to the necessity of a special introduction system for stent insertion through a balloon overtube[38]. Early stent migration is an important drawback and is the main reason of clinical failure, but can be solved using cyanoacrylate, with a

Table 2 Cases series evaluating the outcomes of biodegradable stents with polydioxanone for benign intestinal and colonic strictures

Ref.	Year	Type of study	Indication	*n*	Follow-up (mo)	Technical success	Complications	Clinical success
Janík *et al*[37]	2011	CS	PS	3	5	100%	0%	66%
Rejchrt *et al*[8]	2009	CS	Stricturing CD	11	14	91%	Stent migration, 27%	64%
Toth *et al*[36]	2011	CR	PS	1	5	100%	0%	100%
Pérez Roldán *et al*[35]	2012	CS	PS and fistula	10	13	90%	Stent migration, 10%	50%
Repici *et al*[34]	2013	CS	PS	11	3	100%	Stent migration, 36%	45%
Rodrigues *et al*[39]	2013	CR	Stricturing CD	1	16	100%	0%	100%

CR: Case report; CS: Case series; RCT: Randomized clinical trial; CD: Crohn's disease; PS: Postsurgical strictures.

clip placement in the upper flare or by improvements in stent design. Mucosal hyperplasic reaction after insertion of BDS has been documented in esophageal strictures but not in intestinal strictures.

Pancreatobiliary tract

Endoscopic therapy of benign biliary strictures (BBSs) is now first-line therapy. The paper of BDSs in clinical practice is unknown, and is one the main targets in endoscopic research. The causes of BBSs are diverse, with the 2 most common causes being postsurgical strictures and chronic pancreatitis[40]. Commercially available plastic and metal stents for the bile duct and pancreatic duct have many limitations. Endoscopic placement of BDSs have demonstrated feasibility of implantation, relatively safety, and potential efficacy in the biliary and pancreatic ducts in animal models[41,42]. The use of a novel, self-expanding, radiopaque PLA-barium sulphate BDS and a polyethylene stent was investigated in 12 pigs with cystic-duct leakage, showing that the total external output of bile was significantly smaller with BDS compared with the plastic stent group[42]. This group of authors investigated the degradation, patency, and toxicity of this PLA-BDS stent placed into the pancreatic duct of pigs, and after six months no histological or anatomical changes were observed[41].

Recently, other group of authors have developed other self-expandable BDS made from a poliglecaprone, an absorbable surgical suture. This stent was successfully endoscopically inserted in the pancreatic and bile ducts in 4 pigs[43]. However, future animal studies are needed to evaluate the short-term patency, tissue reactivity and degradation of this stent.

There is no published clinical experience with endoscopic placement of BDSs. However, percutaneously placement of BDSs have been addressed recently[44,45]. In this two pilot studies a total of 12 patients with refractory postsurgical BBSs have been treated with BDSs-PDX in the biliary tree, with clinical success with up to 2-years of follow-up.

CONCLUSION

BDSs are an attractive option to avoid ongoing dilation or surgery in patients with benign stenoses of the small and large intestines. The experience with the currently the only BDS for endoscopic placement, made of PDX, have shown promising results. However some aspects should be improved as are the fact that BDSs lose their radial force over time due to the degradable material, and that can cause stent-induced mucosal or parenchymal injury. This complication rate and modest clinical efficacy has to be carefully considered in individual patients prior to placement of BDSs. Otherwise, the price of these stents therefore it is nowadays an important limitation.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Opioid growth factor and the treatment of human pancreatic cancer: A review

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Abstract

Opioid growth factor (OGF), chemically termed [Met^5]-enkephalin, and its receptor, OGF receptor (OGFr), form a biological axis that tonically regulates cell proliferation by delaying the G_1/S interface of the cell cycle under homeostatic conditions or in neoplasia. Modulation of the OGF-OGFr pathway mediates the course of pancreatic cancer, with exogenous OGF or upregulation of OGFr repressing growth of human pancreatic cancer cells in culture and in nude mice. OGF therapy alone or in combination with standard chemotherapies such as gemcitabine and 5-fluorouracil results in enhanced inhibition of DNA synthesis and tumor growth. Molecular manipulation of OGFr confirms that the receptor is specific for OGF's inhibitory action. Preclinical studies have warranted Phase I and Phase II clinical trials using OGF infusions as a treatment for patients with advanced, unresectable pancreatic cancers. OGF, an endogenous neuropeptide, is a safe, non-toxic, and effective biotherapy that utilizes the OGF-OGFr axis to mediate pancreatic tumor progression.

Key words: Enkephalins; DNA synthesis; Pancreatic adenocarcinoma; Opioids; Nude mice; Receptor transfection

Core tip: Opioid growth factor (OGF) biotherapy for human pancreatic cancer is based on inhibition of DNA synthesis by upregulation of cyclin-dependent inhibitory kinases. Preclinical studies using human pancreatic cancer cell lines have demonstrated that OGF interaction with its selective receptor OGF receptor (OGFr) is a physiological determinant of cell proliferation. Addition of OGF to standard chemotherapies enhances the efficacy of treatment. Studies in nude mice confirm that the OGF-OGFr axis regulates pancreatic cancer progression. Clinical trials using OGF for treatment of patients with unresectable pancreatic tumors reveal that OGF is a novel endogenous opioid that is safe, non-toxic, elicits negligible side effects and reduces pancreatic tumor size in persons who have failed other therapies.

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INTRODUCTION

Novel therapies for human pancreatic cancer are needed to treat the more than 45000 people in the United States who will be diagnosed with this cancer in 2013[1]. Death due to this cancer approaches the same number, with estimates of more than 38000 persons dying of pancre-

atic cancer in 2013. Males have increased incidence and death rates relative to females; black ethnic groups have the highest incidence[1,2]. Five-year survival rates range between 5% and 6%, and have not changed in more than a decade of research[2]. Therapeutics based on underlying mechanisms of disease are needed.

The standard of care for pancreatic cancer is gemcitabine. This anti-metabolite is a nucleoside analog that blocks DNA replication, or inhibits ribonucleotide reductase, an enzyme needed to produce the deoxyribonucleotides necessary for DNA replication; both pathways induce apoptosis and slow tumor growth[3]. However, gemcitabine cannot be used if the patient has allergies (*e.g.*, dye, additives, food), other diseases (*e.g.*, kidney, liver, hepatitis, heart, lung, diabetes, gout), or infections. Radiation therapy cannot be combined with gemcitabine, and women of child-bearing age are encouraged not to take gemcitabine as it may cause birth defects[3].

New endogenous peptide pathways have been identified that provide novel targets for non-toxic therapeutic alternatives for pancreatic cancer. Our knowledge about the biology of pancreatic cancer supports the need for treatments that target the biology of this cancer.

ENDOGENOUS OPIOIDS

An endogenous opioid peptide and its receptor were first identified more than 3 decades ago as being an important inhibitor of human cancer cell proliferation[4,5]. Ongoing studies on the opioid growth factor (OGF)-OGF receptor (OGFr) axis have characterized this pathway and defined mechanistic approaches for the treatment of neoplasia[6]. The peptide is chemically termed [Met^5]-enkephalin, and is a 5-amino acid neuropeptide secreted by the brain and originally identified as an endogenous opioid by scientists in the mid-1970s[7-9]. This peptide was renamed OGF after discoveries of its growth modulating characteristics in mouse neuroblastoma cells and developing rat brain[4,5,10], and to distinguish its pharmacological function from neurotransmission. OGF is derived from both preproenkephalin and pro-opiomelanocortin genes[11], and is rapidly translated and degraded in human blood. Studies have shown that OGF is autocrine and paracrine produced in tissues originating from dermal derivatives, with brain and gut tissues having the greatest levels of the peptide[12,13].

The inhibitory effects of OGF on cell replication were first recorded in developing rat brain[14,15] and in tissue culture studies on mouse and human neuroblastoma[16-19]. OGF inhibits DNA synthesis and cell replication of normal cells and tissues[20,21], human neoplasia[22], and bacteria[23]. The main action of OGF is to upregulate inhibitory kinases in the cell cycle process. OGF's activity is receptor-mediated, dose-related, time-dependent, and reversible. The peptide is present in developing and renewing tissues, and has been localized in embryonic tissues and many human cancers[24-27].

OPIOID RECEPTORS

Classical opioid receptors were discovered in 1973 by three independent laboratories[28-30] and were identified in brain and gastrointestinal tissues. Based on their binding activities to radioisotopes, three classes of receptors - mu (MOR), delta (DOR), and kappa (KOR) were characterized in membrane homogenates as 7-member transmembrane cytoplasmic receptors. The gene and protein structures for the classical receptors are homologous, and many of the opioid ligands cross-react with more than one receptor. OGF binds to DOR and MOR receptors. However, a series of biochemical and pharmacological studies demonstrated that OGF also binds to a new opioid receptor, OGFr, that is located on the nuclear membrane[31-33]. Sequencing of OGFr reveals little or no homology with classical opioid receptors. However, OGFr does display pharmacological characteristics of opioid receptors such as stereospecificity of ligands and opioid antagonist blockade[34]. Subcellular fractionation studies of OGFr in developing rat brain and neuroblastoma cells reveal that OGFr is associated with the nuclear membrane, and immunoelectron microscopy studies have shown that OGF co-localizes with OGFr on the outer nuclear membrane and within the nucleus[35].

OGF-OGFR AXIS: PRECLINICAL *IN VITRO* STUDIES

OGF and OGFr are present in human pancreatic cells (PANC-1, MIA PaCa-2, BxPC-3 and Capan-1) grown in culture, xenografted to nude mice, or surgical specimens obtained during tumor resection[36-39]. *In vitro* studies using PANC-1 cells reveal that the receptor has specific and saturable binding affinity to radiolabeled [Met^5]-enkephalin, with enriched binding in the nuclear fraction of cells[38]. Competition experiments using ligands for classical opioid receptors [*e.g.*, [D-Ala^2, NMe-Phe^4, Gly-ol^5]-enkephalin] (DAMGO), [D-Pen^{2,5}]-enkephalin (DPDPE), dynorphin A1-8, morphine] do not displace the affinity of [Met^5]-enkephalin for OGFr[38] supporting the selectivity of peptide and receptor.

The efficacy of OGF has been characterized in a series of tissue culture studies[37]. OGF inhibits DNA synthesis and the growth of PANC-1 cells in a dose-dependent (42% reductions) and temporal manner (up to 48% reductions at 120 h) relative to control cultures, with its action being receptor-mediated and reversible. Absorption of endogenous ligand using OGF antibodies negated growth inhibition associated with exogenous peptide administration. OGF has a ubiquitous effect on pancreatic cancer cells derived from tumors at different stages of differentiation. Administration of OGF for 72 h inhibits growth (up to 37%) of Capan-1 and MIA PaCa-2 cells, well-differentiated cancer cell lines[40,41], BxPC-3, a moderately well to poorly differentiated cell line[42], as well as PANC-1, an undifferentiated pancreatic cancer cell line[43].

Combinatorial therapeutic regimens are often more

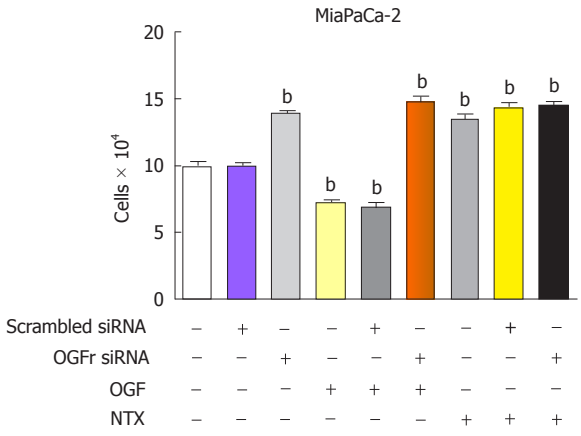

Figure 1 Opioid growth factor receptor is required for the inhibitory action of opioid growth factor and the stimulatory action of NTX on growth of MIA PaCa-2 human pancreatic cell cultures. Cells were transfected with opioid growth factor receptor (OGFr) siRNA or scrambled siRNA for 24 h and then treated with 10^{-6} mol/L. Opioid growth factor (OGF) or NTX, or 100 μL of sterile water for 72 h; compounds and media were replaced daily. Values are expressed as mean ± SE for cell counts from 2 aliquots/well and at least 2 wells/treatment. $^{b}P < 0.001$ *vs* untransfected cultures.

effective than single agent therapy. Gemcitabine is the standard of care for advanced pancreatic neoplasia, and also acts through inhibition of DNA synthesis[3]. Utilizing MIA PaCa-2 cells grown in log phase conditions, the combination of OGF (10^{-6} mol/L) and gemcitabine (10^{-8} mol/L) reduces cell number from control levels by more than 45% over 48 h, whereas each compound alone inhibits growth by less than 22% in the same time period. The action of OGF, but not gemcitabine, is mediated by a naloxone-sensitive receptor and is reversible. Combining OGF with 5-fluorouracil (5-FU; 10^{-6} mol/L) also increases inhibitory action. Over a 96 h period, OGF and 5-FU reduce cell number up to 30%, whereas each compound alone reduces cell number by up to 18%[39].

The specificity of OGF and OGFr has been documented in a variety of experiments using different human pancreatic cancer cell lines. The specificity of OGF has been confirmed by addition of multiple ligands that are specific for classical opioid receptors yet have no effect on cell proliferation and/or growth of cells or tumors[37-39]. Absorption of OGF by antibodies to the endogenous peptide depresses growth, demonstrating the specificity of this peptide. Competition binding assays using classical ligands such as DAMGO, DPDPE, morphine, ethylketocyclazocine and others results in no loss of binding of OGF to OGFr, suggesting little or no affinity of other ligands for OGFr[38].

OGFr selectivity and specificity for the ligand OGF have been shown in several experiments. In tissue culture, siRNA knockdown of RNA and protein expression of OGFr results in cultures that grow faster than controls because there is no receptor available for interaction with endogenous inhibitory OGF. Addition of exogenous OGF to cultures lacking OGFr has no inhibitory action (Figure 1). Finally, transient transfection experiments that knockdown classical opioid receptors using siRNAs to MOR, DOR, or KOR results in no alteration in cell proliferation in homeostatic conditions or following the addition of OGF[44].

IN VIVO STUDIES ON OGF INHIBITION OF PANCREATIC TUMOR GROWTH

Transplantation of BxPC-3 human pancreatic cancer cell lines into nude mice has established a model to study how OGF inhibits tumor incidence and growth[36]. Athymic mice were inoculated subcutaneously with BxPC-3 cells and injected intraperitoneally 3 times daily with 5 mg/kg OGF or sterile saline. OGF-treated mice exhibited a delay of 43% in the time of initial tumor appearance relative to controls (10.6 d). Importantly, 62% of the OGF-treated mice did not have tumors on the day when 100% of all saline-injected mice had visible tumors, suggesting that OGF inhibits proliferation of tumor cells in such a way as to prevent tumor appearance. For those mice receiving OGF that did develop tumors, growth was markedly slower relative to mice injected with saline.

Studies utilizing MIA PaCa-2 cells were conducted in nude mice receiving combination therapy of OGF and/or gemcitabine[39]. Measurement of human pancreatic tumor growth (MIA PaCa-2) in nude mice revealed marked reductions in tumor progression under all treatment modalities, but combination therapy resulted in tumors with marked reductions in size relative to control mice, as well as mice receiving either OGF alone (10 mg/kg daily) or gemcitabine alone (120 mg/kg every 3rd day) (Figure 2). Tumor volumes after 45 d of treatment were reduced approximately 83% in the combination therapy group relative to controls (8900 mm^3), whereas reductions in tumor volume were 45% and 56% for mice receiving OGF alone or gemcitabine alone, respectively, relative to controls.

The relationship between OGFr levels and the progression of human pancreatic tumors in nude mice was investigated by assaying OGFr binding activity and *OGFr* gene expression in tumors of small, medium, or large volume[45]. Binding capacity of OGFr, and transcriptional activity of OGFr, were not dependent on the size of tumor and were unaltered between small and large tumors. Interestingly, OGF plasma levels were decreased up to 7.9-fold in untreated mice with tumors relative to normal, non-tumorigenic mice suggesting that production of the inhibitory peptide, but not the receptor, may be deficient as cancer progresses[45]. These data support that exogenous administration of OGF is important as a therapy because the receptor is present and functioning in late-stage mouse tumors.

Overexpression of OGFr in tumor cells transplanted into nude mice confirmed that the OGF-OGFr axis provides tonic, homeostatic regulatory control of pancreatic neoplasia[46,47]. MIA PaCa-2 cells were stably transfected to overexpress OGFr, selectively cloned and expanded, and inoculated into nude mice; phenotypic changes in tumorigenicity were monitored. Analysis of receptor number showed that transfected tumor tissue had more

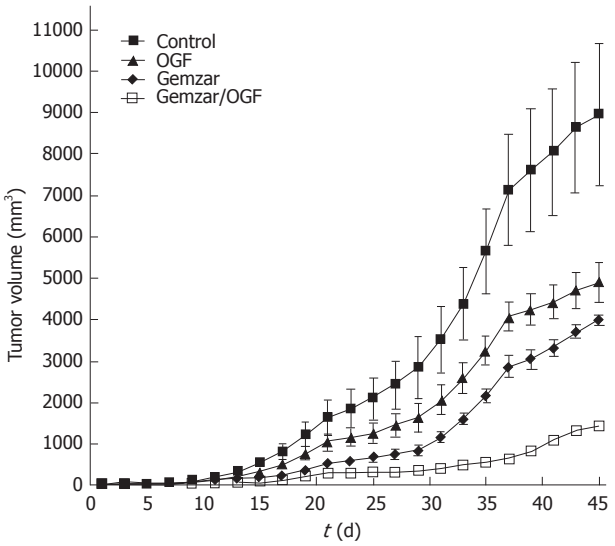

Figure 2 Tumor growth of MIA PaCa-2 tumors xenografted into nude mice. Animals were injected with 10 mg/kg opioid growth factor (OGF) daily, 120 mg/kg gemcitabine every 3 d (gemzar), both OGF and Gemzar, or 0.1 mL of sterile saline daily (control). Tumor volumes were monitored with calipers over a 45-d period of time. Values represent mean ± SE for all mice in the group. See original manuscript[39] for statistical comparisons.

than 4 times the binding capacity compared to wildtype tumors. Tumor incidence in mice receiving the molecularly manipulated cells was reduced up to 50% from animals inoculated with wildtype or empty vector transfected cell lines. Latency for the appearance of a measurable tumor was increased 30%, whereas tumor volumes were decreased 70% in comparison to measurements in mice receiving cells transfected with empty vector cDNA constructs. Treatment of mice with an overexpression of OGFr reduced tumor volumes even more with reductions up to 55% recorded[47]. Therefore, OGFr is a regulator of neoplastic cell proliferation that impacts human pancreatic tumorigenic expression. Modification of receptor number alone may prevent or delay human pancreatic cancer.

OGF-OGFR AXIS: MECHANISM OF ACTION

The mechanism of action of OGF is targeted to DNA synthesis and is directed to the p21 cyclin-dependent inhibitory kinase pathway in human pancreatic cancer[37,48,49]. OGF action is mediated by the receptor OGFr. Unlike the mechanistic pathways of many of the standard chemotherapies, investigations have shown that OGF is nontoxic and does not induce apoptosis[50]. Using a variety of human cancer cell lines, studies have demonstrated that OGF does not reduce cell number by changing other biological pathways associated with migration, differentiation, or cell death[50-52]. Flow cytometric analyses of BxPC-3 cell lines treated with OGF reveal a notable increase in cell number in the G_0/G_1 phase and compensatory reduction in the proportion of cells in the S and G_2/M phases. The percentage of labeled mitotic cells was increased in the G_0/G_1 phase[48]. Further studies utilizing synchronized cultures of BxPC-3 pancreatic cells were directed at deciphering the specific pathway in the cell cycle that is targeted by OGF and focused on the retinoblastoma pathway[49]. It was found that OGF decreased phosphorylation of retinoblastoma protein, but did not change the overall level of retinoblastoma protein. The change was correlated with a reduction in cyclin dependent kinase activity (cdk-2), and increased p21 expression[49]. In general, human pancreatic cancer cells express p21 cyclin-dependent inhibitory kinase pathways, whereas many other cancers (*e.g.*, squamous cell carcinoma of the head and neck) utilize p16 cyclin-dependent inhibitory kinase pathways because of deletions or mutations in the p21 pathway. The presence of one intact pathway is important to maintain a homeostatic balance of cellular replication, allowing for one pathway to be mutated as is often the case in neoplasia. The requirement of an intact OGF-OGFr axis for regulation of pancreatic neoplasia was corroborated in a study[44] whereby more than 30 human cancer cell lines were transfected to repress OGFr cDNA and OGFr expression. The lack of OGFr rendered OGF ineffective in inhibiting proliferation.

CLINICAL STUDIES ON THE SAFETY AND EFFICACY OF OGF FOR TREATMENT OF HUMAN PANCREATIC CANCER

Preclinical studies on OGF have shown no toxicity and significant efficacy toward repressing pancreatic cancer progression. Clinical trials to assess OGF treatment of advanced pancreatic cancer were conducted by Zagon *et al*[52] and Smith *et al*[53] at The Pennsylvania State University College of Medicine. The maximum tolerated dose (MTD) was established at 250 μg/kg infused over a period of 30 min[52]. Patients with unresectable advanced pancreatic adenocarcinoma were treated with the MTD to establish safety and toxicity. No adverse effects related to cardiac rhythm, blood values, neurological status or other laboratory tests were reported; hypotension was the dose-limiting toxicity. Of interest were the signs of efficacy shown by the small number of patients in this phase I trial. Mean survival time for the patients in the study, including those receiving only one dosage of OGF, was over 8.5 mo, and two patients had resolution of liver metastases. These observations support further clinical trials on OGF as a treatment of advanced pancreatic cancer.

A prospective phase Ⅱ open-labeled clinical trial with 24 patients who failed standard chemotherapy for advanced pancreatic cancer was conducted whereby patients were treated weekly with 250 μg/kg OGF by intravenous infusion[54]. Outcomes were tumor size measured by computer tomography, survival time, and quality of life. Blood samples were evaluated for levels of OGF after 4 and 8 wk of infusion. Data on the OGF treatment were compared to results obtained from a control group (n-166) of patients of equivalent age who failed therapy

and were discharged to hospice care. OGF-treated patients had a three-fold increase in median survival time in comparison to untreated patients. Tumor size was stabilized or reduced in 62% of the cancer patients receiving OGF and surviving more than 8 wk in order to conduct the tomography. Plasma enkephalin levels were significantly increased at 4 and 8 wk with blood levels reaching approximate 55 pg/mL in comparison to baseline values of 8 pg/mL. Finally, no adverse effects on blood chemistry were noted, confirming the safety and lack of toxicity of OGF. Feedback from patients receiving OGF and their caregivers on quality of life indicated that OGF infusion did not indicate any stress or pain.

OGF BIOTHERAPY

Preclinical studies using a variety of human pancreatic adenocarcinoma cell lines that represent undifferentiated to well differentiated pancreatic neoplasms have demonstrated that OGF inhibits DNA synthesis and cell proliferation *in vitro*. The action of OGF is mediated by OGFr, is reversible, and does not involve apoptotic pathways. OGF is an endogenous peptide that is readily degraded, without alteration of cell migration, differentiation, or survival and thus can be considered a biotherapy. The specificity and selectivity of the OGF-OGFr axis substantiate that this axis is a determinant of cell proliferation in a variety of human cancers.

Investigations of the OGF-OGFr axis in mouse models of cancer with human cell lines transplanted into nude mice confirmed and extended tissue culture studies. Exogenous OGF repressed tumor progression under all situations, and tumors grown from cells overexpressing OGFr were inhibited in their growth. Combination OGF and chemotherapy provided enhanced efficacy at reducing tumor size.

Clinically, OGF is a safe, non-toxic biotherapy that extends survival and reduces tumor burden in patients with unresectable pancreatic cancer. In summary, the OGF-OGFr axis should be explored both as a primary therapy for pancreatic cancer, and as an adjuvant pathway with other chemotherapies.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Beyond first-line chemotherapy for advanced pancreatic cancer: An expanding array of therapeutic options?

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Abstract

While an increasing number of therapeutic options are now available for the first-line treatment of locally advanced or metastatic pancreatic cancer, the optimal choice for treatment in the second-line setting and beyond is less well defined. A variety of cytotoxic agents, either alone or in combination, have been evaluated, although primarily in the context of small single-arm or retrospective studies. Most regimens have been associated with median progression-free survival rates in the range of 2-4 mo and overall survival rates between 4-8 mo, highlighting the very poor prognosis of patients who are candidates for such treatment. Targeted therapies studied in this chemotherapy-refractory setting, meanwhile, have produced even worse efficacy results. In the current article, we review the clinical evidence for treatment of refractory disease, primarily in patients who have progressed on front-line gemcitabine-based chemotherapy. In the process, we highlight the limitations of the available data to date as well as some of the challenges in designing appropriate clinical trials in this salvage setting, including how to select an appropriate control arm given the absence of a well-established reference standard, and the importance of incorporating predictive biomarkers and quality of life measures whenever possible into study design.

Key words: Pancreatic cancer; Refractory; Second-line chemotherapy; Gemcitabine

Core tip: No standard of care exists for patients with advanced pancreatic cancer who have progressed on front-line chemotherapy. To date, most available evidence has come from small non-randomized studies, with efficacy results that have been fairly dismal. In this review, we discuss both traditional and novel cytotoxic and targeted therapies that have been evaluated in this refractory setting and how they may (or may not) be applicable to clinical practice; and raise considerations for clinical trial design in the future, particularly in this current era of both expanding chemotherapeutic options and molecular/"precision" medicine.

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INTRODUCTION

More than 80% of patients diagnosed with pancreatic adenocarcinoma have metastatic or locally advanced inoperable disease at the time of initial presentation[1], at which point systemic therapy becomes the mainstay of care. Over the past decade-plus, gemcitabine alone or in combination with other drugs (most commonly a fluoropyrimidine, a platinum analogue, or the epidermal growth

factor receptor inhibitor erlotinib) have represented the most commonly used front-line treatment options. The treatment landscape is gradually shifting, however, with recent positive results from a couple of phase Ⅲ studies establishing two new standards of care for first-line treatment, FOLFIRINOX [infusional 5-fluorouracil (FU), leucovorin, irinotecan, oxaliplatin] and the doublet of gemcitabine plus *nab*-paclitaxel.

Invariably, regardless of choice of front-line therapy, patients with advanced/metastatic disease will progress, and at that point the choice of treatment becomes considerably murkier. According to results from one United States cooperative group trial (CALGB 80303), fewer than half of patients with advanced pancreatic cancer went on to receive any additional therapy after progressing on front-line study treatment[2]. This reflects, in part, the fact that patients in this setting frequently demonstrate significant clinical deterioration and a decline in performance status, and are no longer deemed appropriate candidates for further anti-cancer therapy. However, it also highlights the fact that no second-line regimen(s) has consistently and unequivocally been shown to confer a survival benefit for patients, and as such providers are left grasping for best available evidence to inform treatment decisions, especially for patients who wish to remain proactive with some form of therapy.

In this review, we summarize the various therapeutic options that have been evaluated to date in the second-line (and beyond) setting for advanced pancreatic cancer. In so doing, we raise a number of important issues regarding appropriate clinical trial design, what (if any) should be considered a correct reference standard and benchmark of success in this setting, and how the expanding armamentarium of available agents and established regimens for this disease both expands our array of therapeutic options and adds to the complexity in decision-making.

GEMCITABINE-CONTAINING REGIMENS

Gemcitabine emerged as the standard of care for first-line treatment of advanced pancreatic cancer following its FDA approval in 1996[3]. Once patients develop resistance following front-line gemcitabine-based therapy, the natural question arises as to whether continuing with this same drug while adding novel agents can confer, or restore, clinical activity by overcoming drug-specific chemotherapeutic resistance and/or through synergistic effects.

Kozuch *et al*[4] first demonstrated the feasibility of this approach in a retrospective analysis of 34 consecutive patients with metastatic pancreatic cancer receiving irinotecan/gemcitabine/5-FU/leucovorin/cisplatin (G-FLIP), 32 of whom had previously progressed on gemcitabine and 31 who had progressed specifically on gemcitabine/5-FU/cisplatin (GFP). Of these 31 patients, whose regimen was altered only by the addition of irinotecan, 7 (23%) achieved partial responses (PR) and 7 (23%) achieved stable disease (SD). Notably, 8 of these 14 patients demonstrating disease control had previously experienced progressive disease as a best response to GFP alone. Median progression-free and overall survival (OS) for all 34 patients receiving second-line G-FLIP was 3.9 and 10.3 mo, respectively.

Another multidrug regimen that has been evaluated in the refractory setting is cisplatin/epirubicin/5-FU/gemcitabine (PEFG). This combination was initially tested in the front-line setting in an Italian phase Ⅲ trial by Reni *et al*[5], and showed improved 4-mo PFS and 2-year survival rates compared to gemcitabine monotherapy, albeit with significant rates of hematologic toxicity. PEFG was subsequently studied by the same research group as second-line therapy in patients with progressive or metastatic disease refractory to gemcitabine-based treatment. In this 46-patient study, subjects receiving either classic or dose-intense PEFG had a median OS of 8.3 mo, with no significant difference between the different doses of PEFG tested[6]. Again, marked toxicities were noted, including Grade 3-4 neutropenia and thrombocytopenia in 26 (56%) and 10 (22%) patients, respectively.

Building upon observations from prior phase Ⅲ trials demonstrating improvements in response rate (RR), progression free survival (PFS), and clinical benefit response (CBR) of gemcitabine/platinum doublets compared to gemcitabine monotherapy in the front-line setting[7,8], a similar strategy has also been explored in the gemcitabine-refractory setting in a variety of contexts. Demols *et al*[9] investigated the combination of gemcitabine plus oxaliplatin (GemOx) in a single-arm phase Ⅱ study involving 33 patients with gemcitabine-refractory advanced pancreatic cancer. A partial response was observed in 7 patients (21%) with an additional 12 patients (36%) achieving SD. Median OS was 6 mo. Importantly, 17 patients (52%) were reported as having a clinical benefit response. One more recent approach has involved testing the potential for enhanced chemotherapeutic efficacy at higher temperatures[10], by which basis Tschoep-Lechner *et al*[11] conducted a study of gemcitabine and cisplatin combined with regional hyperthermia (RHT) in the second-line setting. Median time to progression for the 23 patients treated with this strategy was 4.3 mo, with a median overall survival of 12.9 mo. These results have spurred an ongoing prospective phase Ⅱ trial offering second-line Gem/Cis/RHT (EudraCT: 2005-003855-11).

Other doublet regimens that have been evaluated in the salvage setting include gemcitabine plus the oral fluoropyrimidine S-1[12] and gemcitabine plus *nab*-paclitaxel[13] with median times to progression of 2.8 and 3.2 mo, respectively. More details of these and other gemcitabine-based combinations are summarized in Table 1.

NOVEL MONOTHERAPEUTIC REGIMENS

An alternative approach to second-line therapy involves administration of a completely non-cross-resistant regimen; using such a strategy, previous agents (such as

Table 1 Clinical studies of second-line gemcitabine-containing regimens

Ref.	Regimen	Sample size	RR[1]	PFS/TTP (mo)	Med OS (mo)	1 yr survival
Kozuch *et al*[4], 2001	G-FLIP	34	24%	3.9	10.3	47%
Reni *et al*[6], 2008	PEFG	46	24%	5.0	8.3	26%
Demols *et al*[9], 2006	GEMOX	33	21%	4.2	6.0	NR
Fortune *et al*[76], 2009	GEMOX	17	24%	2.6	6.4	29%
Stathopoulos *et al*[77], 2006	Gem, Lipoplatin	24	8.3%	NR	4.0	NR
Tschoep *et al*[11], 2013	Gem, Cisplatin, RHT	23	4.3%	4.3	NR	NR
Morizane *et al*[12], 2012	Gem, S-1	40	18%	2.8	7.0	18%
Ernani *et al*[13], 2012	Gem, *nab*-Paclitaxel	10	20%	3.2	NR	NR

[1]Intent-to-treat analysis. G-FLIP: Gemcitabine, 5-fluorouracil, leucovorin, cisplatin; PEFG: Cisplatin, epirubicin, 5-fluorouracil, gemcitabine; GEMOX: Gemcitabine, oxaliplatin; Gem: Gemcitabine; RHT: Regional hyperthermia; *Nab*-paclitaxel: Albumin-bound nanoparticle paclitaxel; NR: Not reported; PFS: progression free survival; OS: Overall survival; TTP: Time to progression.

gemcitabine) are discontinued and an entirely new drug or drug combination is given. In terms of monotherapy, several topoisomerase inhibitors have been investigated in patients refractory to gemcitabine-based front-line treatment. The orally active camptothecin rubitecan, for example, showed sufficient single-agent activity in two separate studies of gemcitabine-refractory disease[14,15] to warrant a randomized phase Ⅲ trial in which 409 pretreated patients (70% of whom had received two or more prior regimens) were randomized to receive either rubitecan monotherapy or "best choice (BC)" alternative therapy as determined by treating physicians (most commonly gemcitabine, 5-FU, mitomycin C, capecitabine, or docetaxel). Presented as an abstract at the 2004 ASCO annual meeting but never subsequently published, the trial did not show a statistically significant difference in overall survival between groups (108 d *vs* 94 d, respectively, $P = 0.63$), although significant improvements were observed with rubitecan in terms of progression-free survival (58 d *vs* 48 d, $P = 0.01$) and response rate (6.1% *vs* 0.5%, $P = 0.01$)[16].

More recently, a phase Ⅱ study of liposomal irinotecan sucrosofate (PEP02, MM-398), a drug formulation with improved pharmacokinetics and tumor bioavailability relative to free irinotecan, was performed in patients with metastatic pancreatic cancer refractory to front-line gemcitabine-based therapy[17]. Ko *et al*[17] reported a disease control rate of 50% (including 7.5% with an objective response) as well as a 50% or greater CA19-9 decline in 31% of evaluable subjects, with a median overall survival of 5.2 mo. Toxicities were manageable, with cytopenias, asthenia, and diarrhea representing the most common grade 3/4 adverse events. These results prompted the launch of an international randomized phase Ⅲ trial (NAPOLI-1, NCT01494506) that has been recently completed, comparing MM-398 with or without 5-FU/leucovorin to 5-FU/leucovorin alone.

Inhibitors of microtubule dynamics, including taxanes (docetaxel, paclitaxel, *nab*-paclitaxel) and eribulin mesylate, have also been investigated in small retrospective and single-arm phase Ⅱ studies[18-22]. Given the unique formulation of *nab*-paclitaxel that may allow it to more successfully traverse the blood-stroma barrier, in addition to the positive results from the phase Ⅲ MPACT trial establishing the combination of *nab*-paclitaxel/gemcitabine as a viable option for first-line therapy[23], there has been natural interest in evaluating this agent in the salvage setting. To date, we only have results from a small phase Ⅱ study of *nab*-paclitaxel as a single agent for refractory pancreatic cancer, in which there was a single objective response (with an additional 6 achieving disease stabilization) amongst 19 patients, with a median PFS of 1.7 mo. Estimated median OS in this cohort was 7.3 mo[22].

Fluoropyrimidines have also been studied in the advanced refractory disease setting. Boeck *et al*[24] studied second-line capecitabine monotherapy after gemcitabine failure and observed disease stabilization in 39% of patients (no objective responses), with a median time to progression and overall survival of 2.3 mo and 7.6 mo, respectively. Another oral fluoropyrimidine, S-1, widely used in Asia and other parts of the world for gastric and pancreatic cancer, has also been evaluated in several phase Ⅱ studies as monotherapy for gemcitabine-refractory patients; response rates associated with this agent range from 4%-15%, with a median PFS almost uniformly in the 2 mo range[25-28]. See Table 2 for additional data from these studies.

CYTOTOXIC COMBINATION REGIMENS (NON-GEMCITABINE-BASED)

Patients who maintain a good performance status after progressing on front-line therapy may also be candidates for non-gemcitabine-based combination chemotherapy regimens.

Platinum-based combinations

To date, the majority of studies have concentrated on the combination of a fluoropyrimidine plus a platinum analogue, most notably 5-FU, leucovorin, and oxaliplatin administered in various dosing schedules. One of the earliest studies, a non-randomized phase Ⅱ trial conducted in Greece by Tsavaris *et al*[29], showed encouraging clinical activity of these drugs when administered weekly in bolus fashion, with the best response including partial responses in 7 of 30 patients (23%) and stable disease in an additional 9 (30%). More traditional FOLFOX regimens, with biweekly dosing schedules and prolonged 5-FU infusion

Table 2 Clinical studies of second-line monotherapeutic regimens

Ref.	Regimen	Sample size	RR[1]	PFS/TTP (mo)	Med OS (mo)	1 yr survival
Jacobs *et al*[16], 2004	Rubitecan	198	11%	1.9	3.5	NR
Burris *et al*[15], 2005	Rubitecan	58	5.2%	2.0	3.1	9%
Yi *et al*[78], 2009	Irinotecan	33	9%	2.0	6.6	NR
Takahara *et al*[79], 2013	Irinotecan	56	3.6%	2.9	5.3	NR
Ko *et al*[17], 2013	Nanoliposomal irinotecan	40	7.5%	2.4	5.2	25%
Oettle *et al*[18], 2000	Paclitaxel	18	5.6%	NR	4.1	NR
Maeda *et al*[19], 2011	Paclitaxel	30	10%	NR	6.7	NR
Cereda *et al*[20], 2008	Docetaxel	10	0%	1.5	4.0	0%
Hosein *et al*[22], 2013	*Nab*-Paclitaxel	19	5%	1.7	7.3	37%
Boeck *et al*[24], 2007	Capecitabine	39	0%	2.3	7.6	NR
Bodoky *et al*[59], 2012	Capecitabine	38	7.9%	2.2	5.0	NR
Morizane *et al*[25], 2009	S-1	40	15%	2.0	4.5	14%
Todaka *et al*[26], 2010	S-1	52	3.8%	2.1	5.8	12%
Mizuno *et al*[28], 2013	S-1	67	6%	1.9	5.9	NR
Ioka *et al*[27], 2013	Best fluoropyrimidine[2]	40	10%	3.8	7.5	NR
Fukahori *et al*[80], 2012	Gemcitabine[3]	27	14%	2.6	8.0	NR
Androulakis *et al*[81], 2005	Oxaliplatin	18	0%	NR	3.5	NR
Boeck *et al*[82], 2007	Pemetrexed	52	3.8%	1.6	4.7	NR
Ulrich-Pur *et al*[48], 2003	Raltitrexed	19	0%	2.5	4.3	0%
Kindler *et al*[83], 2008	Arsenic trioxide	13	0%	1.6	3.8	0%

[1]Intent-to-treat analysis; [2]S-1 (67.5%), uracil-tegafur (20%), or 5-fluorouracil (12.5%); [3]S-1 refractory disease. *Nab*-paclitaxel: Albumin-bound nanoparticle paclitaxel; NR: Not reported; PFS: Progression free survival; OS: Overall survival; TTP: Time to progression.

times similar to that given in colorectal cancer, have also been examined with demonstrable evidence of activity in this setting. Yoo *et al*[30] conducted a randomized phase Ⅱ trial comparing modified versions of FOLFOX and FOLFIRI (5-FU, leucovorin, irinotecan) for gemcitabine-refractory advanced pancreatic cancer. However, in this study, response rates to both regimens were low (7% and 0%) with associated PFS times of 6.0 and 8.3 wk, respectively. A more recent phase Ⅱ trial of FOLFOX4 from Korea reported modestly better results, with an objective response rate of 11%, a tumor stabilization rate of 41%, and a median time to progression of 9.9 wk[31]. Single-arm studies of capecitabine plus oxaliplatin (CapOx) have also been performed by several Asian groups, with fairly comparable results[32-35].

The most convincing evidence supporting a fluoropyrimidine/platinum-based combination comes from Germany, using a regimen termed OFF, in which 5-FU (given as a 24-h infusion) plus folinic acid are given weekly x 4 in 6-wk cycles, with the addition of oxaliplatin during weeks 2 and 4. Prompted by promising results from a phase Ⅱ trial using this regimen (disease control rate lasting 12 wk or better in 43% of study patients), a phase Ⅲ randomized trial was designed by Charité Onkologie (CONKO-003) in which patients were randomized to receive either the OFF regimen or best supportive care (BSC). A sample size of 165 was planned, but the study was stopped due to poor accrual (likely from the possibility of randomization to a BSC arm) after enrolling 46 patients[36]. Even with the limited sample size, overall survival in patients receiving OFF was 4.8 mo compared to 2.3 mo in those receiving BSC (P = 0.008)[37]. The investigators sought to build on these results with another randomized phase Ⅲ trial comparing OFF to weekly 5-FU/folinic acid (FF) alone. The results of this 168-patient trial were presented in abstract form at the 2008 ASCO meeting[38]. As compared to the FF regimen, patients receiving OFF demonstrated improved PFS (13 wk *vs* 9 wk, P = 0.012) and median OS (26 wk *vs* 13 wk, P = 0.014). This trial marks the largest phase Ⅲ study to date showing a survival benefit of second-line therapy for pancreatic cancer; as such, the OFF regimen (or iterations thereof) has become accepted as the de facto standard treatment of refractory disease.

With the emergence of FOLFIRINOX (5-FU, leucovorin, irinotecan, and oxaliplatin) as a front-line standard for patients with advanced pancreatic cancer and good performance status[39], there has naturally been interest in investigating this regimen in the second-line setting. To date, we only have data from one small retrospective series that included 27 patients[40]. Seventeen (63%) demonstrated stable disease or better, including 5 with partial responses, with an associated median TTP of 5.4 mo. Importantly, treatment was generally well-tolerated with manageable and predictable toxicities. Further evaluation of this regimen clearly needs to be performed in prospectively designed studies.

While fluoropyrimidine/platinum combinations have been studied most extensively, single-arm studies of platinum-based agents partnered with other classes of agents, including oxaliplatin in combination with irinotecan[41,42], raltitrexed[43], and pemetrexed[44], have also been examined. Results of these small series are shown in Table 3.

Non-platinum-based combinations

In addition to the previously described phase Ⅱ trial by Yoo *et al*[30] in which gemcitabine-refractory patients were randomized to receive modified versions of either FOLFOX or FOLFIRI, other smaller prospective and retrospective studies of FOLFIRI have been conducted,

Table 3 Clinical studies of second-line cytotoxic combination regimens

Ref.	Regimen	Sample size	RR[1]	PFS/TTP (mo)	Med OS (mo)	1 yr survival
	Platinum based regimens					
Tsavaris *et al*[29], 2005	FOLFOX	30	23%	5.1	5.8	NR
Mitry *et al*[84], 2006	FOLFOX	18	0%	0.9	1.3	NR
Gebbia *et al*[85], 2007	FOLFOX	42	14%	4	6.7	NR
Novarino *et al*[86], 2009	FOLFOX	23	0%	2.7	4.0	NR
Yoo *et al*[30], 2009	FOLFOX	30	6.7%	1.4	3.5	NR
Chung *et al*[31], 2013	FOLFOX	44	11%	2.3	7.3	NR
Berk *et al*[35], 2012	FOLFOX	46	17%	3.7	5.8	NR
Sancho *et al*[32], 2008	CapOx[2]	18	5.6%	3.9	5.8	NR
Xiong *et al*[33], 2008	CapOx	41	2.4%	2.3	5.4	21%
Gasent-Blesa *et al*[34], 2009	CapOx	15	6.7%	NR	5.3	NR
Berk *et al*[35], 2012	CapOx	39	18%	3.7	4.9	NR
Pelzer *et al*[87], 2009	OFF	37	5.4%	2.8	5.1	NR
Pelzer *et al*[37], 2011	OFF	23	0%	NR	4.8	NR
Pelzer *et al*[38], 2008	OFF	76	NR	3	6.1	NR
Assaf *et al*[40], 2011	FOLFIRINOX	27	19%	5.4	8.5	NR
Togawa *et al*[88], 2007	Cisplatin, S-1	17	29%	NR	10	32%
Kim *et al*[89], 2012	Cisplatin, S-1	11	0%	1.5	2.7	NR
Takahara *et al*[90], 2013	Oxaliplatin, S-1	30	10%	3.4	5.0	NR
Cantore *et al*[41], 2004	Oxaliplatin, irinotecan	30	10%	4.1	5.9	23%
Oh *et al*[42], 2010	Oxaliplatin, irinotecan	14	21%	1.4	4.1	7.1%
Reni *et al*[43], 2006	Oxaliplatin, raltitrexed	41	24%	1.8	5.2	12%
Mazzer *et al*[44], 2009	Oxaliplatin, pemetrexed	16	56%	3.3	NR	NR
	Non-platinum based regimens					
Yoo *et al*[30], 2009	FOLFIRI	31	0%	1.9	3.9	NR
Gebbia *et al*[45], 2010	FOLFIRI	40	15%	3.7	6.0	0%
Cereda *et al*[91], 2010	FOLFIRI or XELIRI	34	0%	2.0	4.2	5.6%
Zaniboni *et al*[46], 2012	FOLFIRI	50	8%	3.2	5.0	NR
Neuzillet *et al*[47], 2012	FOLFIRI	63	7.9%	3.0	6.6	NR
Mizuno *et al*[28], 2013	S-1, irinotecan	60	18%	3.6	6.9	NR
Blaya *et al*[49], 2007	Capecitabine, docetaxel	24	13%	NR	NR	NR
Katopodis *et al*[50], 2011	Capecitabine, docetaxel	31	9.7%	2.4	6.4	15%
Kim *et al*[51], 2009	5-FU, paclitaxel	28	10%	2.5	7.6	NR
Lee *et al*[92], 2009	Conti-FAM[3]	31	12%	2.3	6.7	NR
Shi *et al*[93], 2012	Capecitabine, thalidomide	31	6.5%	2.7	6.1	NR
Saif *et al*[94], 2009	Capecitabine, PHY906	25	5.3%	NR	NR	NR
Ulrich-Pur *et al*[48], 2003	Irinotecan, raltitrexed	19	16%	4.0	6.5	NR
Reni *et al*[95], 2004	MDI	15	0%	1.7	6.1	0%
Cereda *et al*[96], 2011	Mitomycin, ifosfamide	21	4.8%	1.7	3.7	9.5%
Ko *et al*[52], 2008	Irinotecan, docetaxel	14	0%	1.2	4.5	21%

[1]Intent-to-treat analysis; [2]Pooled analysis of pancreatic (50%), biliary (22%), gallbladder (22%) and ampullary (6%) cancer; [3]Pooled analysis of pancreatic (48%), biliary (35%) and gallbladder (16%) cancer. FOLFOX: Oxaliplatin, 5-fluorouracil, folinic acid, biweekly; CapOx: Capecitabine, oxaliplatin; OFF: Oxaliplatin, 5-fluorouracil, leucovorin, in 6-wk cycles; FOLFIRINOX: Oxaliplatin, leucovorin, 5-fluorouracil, irinotecan; FOLFIRI: 5-Fluorouracil, leucovorin, irinotecan; XELIRI: Capecitabine, irinotecan; 5-FU: 5-Fluorouracil; Conti-FAM: 5-Fluorouracil, doxorubicin, mitomycin-c; MDI: Mitomycin, docetaxel, irinotecan; NR: Not reported; PFS: Progression free survival; OS: Overall survival; TTP: Time to progression.

with response rates ranging between 8%-15% and median progression-free survival in the 3-4 mo range[45-47]. Another fluoropyrimidine/irinotecan combination termed IRIS (irinotecan plus S-1) was compared to S-1 alone in a randomized phase Ⅱ trial from Japan of 127 patients who had progressed on gemcitabine[28]. The combination produced a response rate of 18%, compared to 6% with S-1 alone (P = 0.03). Median PFS and OS also favored the IRIS combination, although these improvements did not reach statistical significance (107 and 208 d, compared to 58 and 176 d for S-1, respectively). Irinotecan has also been tested in combination with the folate antimetabolite raltitrexed in a randomized phase Ⅱ trial *vs* raltitrexed monotherapy[48]. In this 38-patient study, the doublet was associated with a higher rate of objective response (16% *vs* 0%) and prolonged PFS (4.0 mo *vs* 2.5 mo) and OS (6.5 mo *vs* 4.3 mo), albeit with higher rates of clinically relevant toxicities including gastrointestinal symptoms and alopecia.

Taxanes represent the other most frequently studied class of agents evaluated in the salvage setting for pancreatic cancer. Combination regimens including capecitabine/docetaxel[49,50] and 5-FU/paclitaxel[51] have been studied in small phase Ⅱ trials, with response rates in the 10% range and median PFS centered around 2 mo. A small phase Ⅱ study looking at the combination of irinotecan/docetaxel was discontinued early due to excess toxicity, with no responses observed in 14 evaluable patients[52]. Table 3 highlights other non-platinum-based combinations that have been explored, mostly in the context of single-arm phase Ⅱ studies.

Table 4 Ongoing randomized phase Ⅱ/Ⅲ trials of refractory pancreatic cancer chemotherapy

Clinical trial	Design	Study arms	Goal enrollment	Primary measure	Previous therapy	Status
NCT00674973	Phase Ⅱ	Erlotinib *vs* placebo	207	PFS, biomarkers	1 prior CT regimen	Active, not recruiting
NCT01074996	Phase Ⅱ	S-1 *vs* S-1, leucovorin	96	OS	Gem-based	Recruiting
NCT01417000	Phase Ⅱ	GVAX pancreas, cyclophosphamide, CRS-207 *vs* GVAX pancreas, cyclophosphamide	90	OS	≥ 1 prior CT regimen	Active, not recruiting
NCT01423604	Phase Ⅱ	Capecitabine, ruxolitinib *vs* capecitabine, placebo	138	OS	Gem-based	Active, not recruiting
NCT01658943	Phase Ⅱ	Selumetinib, MK2206 *vs* FOLFOX	133	OS, PFS	Gem-based	Recruiting
NCT01796782	Phase Ⅱ	QYHJ granules *vs* Capecitabine	60	OS	Non-capecitabine containing CT	Active, not recruiting
NCT01121848	Phase Ⅲ	Capecitabine or 5-FU, leucovorin *vs* XELOX or mFOLFOX-6	128	PFS	Gem-based	Active, not recruiting
NCT01494506	Phase Ⅲ	MM-398 *vs* MM-398, 5-FU, leucovorin *vs* 5-FU, leucovorin	405	OS	Gem-based	Active, not recruiting
NCT01954992	Phase Ⅲ	Glufosfamide *vs* 5-FU	480	OS	Gem-based	Recruiting
NCT01956812	Phase Ⅲ	Gemcitabine, IMMU-107 *vs* Gemcitabine, placebo	440	OS	2 prior CT regimens, ≥ 1 Gem-based	Not yet open for recruitment

GVAX pancreas: Allogeneic pancreatic cancer cell vaccine, induces GM-CSF production; CRS-207: Attenuated listeria monocytogenes vaccine, induces immune response to mesothelin; MK2206: Akt inhibitor; FOLFOX: 5-Fluorouracil, leucovorin, oxaliplatin; QYHJ: Qingyihuaji formulation; 5-FU: 5-Fluorouracil; XELOX: Capecitabine, oxaliplatin; mFOLFOX-6: Modified schedule 5-fluorouracil, leucovorin, oxaliplatin; MM-398: Liposomal irinotecan; IMMU-07: Yttrium-90 radiolabeled humanized monoclonal antibody against mucin1 (CD227); PFS: Progression free survival; OS: Overall survival; CT: Chemotherapy; Gem-based: Gemcitabine-containing chemotherapy regimen.

TARGETED THERAPIES

In recent years, an improved understanding of cancer biology has led to the development of targeted therapies intended to inhibit tumor-specific proteins or pathways instrumental in cellular proliferation and survival. These include small molecule inhibitors, which inhibit a specific intracellular protein or pathway; or engineered antibodies, designed to target proteins expressed preferentially on the tumor cell surface. In pancreatic cancer, a number of potentially actionable oncogenic pathways have been identified for which such targeted therapies have been developed and tested, many in the chemo-refractory setting, either alone or in combination with other targeted or cytotoxic agents.

Small molecule inhibitors that bind the intracellular tyrosine kinase (TK) domain of the human epidermal growth factor receptor (HER1/EGFR) block signaling through this pathway that controls aspects of DNA synthesis, cell proliferation, adhesion, and migration. Erlotinib, one such anti-EGFR TK inhibitor (TKI), was approved in the front-line setting for advanced pancreatic cancer based on a small but statistically significant improvement in median survival when added to gemcitabine in a randomized phase Ⅲ trial led by the National Cancer Institute of Canada[53]. When tested as monotherapy in the setting of gemcitabine-refractory disease in a (non-published) phase Ⅱ trial, erlotinib produced prolonged disease control (greater than 8 wk) in 10/40 evaluable patients, with a median time to progression of 1.6 mo and a median survival of 4.1 mo[54]. A randomized trial of erlotinib *vs* placebo (NCT00674973) has completed accrual with the goal of identifying biomarkers predictive of benefit to this agent (Table 4); data are not yet available. Another phase Ⅱ study tested erlotinib in combination with capecitabine in the refractory setting and produced somewhat better results, including a 10% objective response rate, a median PFS of 3.4 mo, and a median OS of 6.5 mo, with no associated grade 4 toxicities[55].

Downstream of EGFR is the protein encoded by the *KRAS* oncogene, which is mutated and hence constitutively activated in the vast majority of pancreatic cancers[56-58]. While KRAS itself has proved to be challenging as a druggable target, KRAS effector pathways such as the MAP (RAF/MEK/ERK) signaling cascade may be more amenable to pharmacologic inhibition. Bodoky *et al*[59] investigated selumetinib, a selective MEK1/2 inhibitor, in a randomized phase Ⅱ trial *vs* capecitabine for gemcitabine-resistant pancreatic cancer. Selumetinib, though well tolerated, did not improve survival relative to capecitabine monotherapy, with median PFS and OS times of 2.1 and 5.4 mo compared to 2.2 and 5.0 mo, respectively. Two of 32 patients on the selumetinib arm (6.3%) did achieve a (unconfirmed) partial response.

Several lines of preclinical evidence indicate that inhibition of MEK induces compensatory hyperactivation of a semi-parallel EGFR signaling pathway, the PI3K/AKT cascade[60], and that simultaneous blockade of multiple nodes leads to better anti-tumor activity. Ko *et al*[61] tested this approach of dual inhibition for refractory pancreatic cancer in a multicenter phase Ⅱ study, using the combination of selumetinib plus erlotinib. Although no objective responses were observed, 12 of 46 patients (26%) achieved stable disease for a minimum of 12 wk, and 38% of evaluable patients had a biomarker response (CA19-9 decline > 50%). Median OS on this study was 7.5 mo. An ongoing randomized phase Ⅱ study led by the Southwest Oncology Group (SWOG 1115) is comparing the combination of selumetinib plus the AKT inhibitor MK2206 to standard FOLFOX chemotherapy in patients who have progressed on front-line gemcitabine-based treatment (NCT01658943) (Table 4).

Table 5 Clinical studies of second-line targeted therapies

Ref.	Regimen	Sample size	RR[1]	PFS/TTP (mo)	Med OS (mo)	1 yr survival
Ignatiadis *et al*[97], 2006	Gefitinib, docetaxel	26	0%	2.1	2.9	NR
Brell *et al*[98], 2009	Gefitinib, docetaxel	41	2.4%	1.8	4.5	0%
Kulke *et al*[55], 2007	Erlotinib, capecitabine	30	10%	3.4	6.5	26%
Tang *et al*[54], 2009	Erlotinib	50	0%	1.6	4.1	6 m = 39%[3]
Iyer *et al*[99], 2010	Erlotinib	18	0%	1.4	3.1	NR
Bodoky *et al*[59], 2012	Selumetinib	32	6.3%	2.1	5.4	NR
Ko *et al*[61], 2013	Selumetinib, erlotinib	46	0%	2.6	7.5	NR
Wolpin *et al*[62], 2009	Everolimus	33	0%	1.8	4.5	NR
Garrido-Laguna *et al*[63], 2010	Sirolimus	31	0%	NR	NR	6 m = 26%[3]
Javle *et al*[64], 2010	Everolimus, erlotinib	16	0%	1.6	2.9	NR
Javle *et al*[64], 2010	Temsirolimus	5	0%	0.6	1.5	NR
Dragovich *et al*[68], 2008	Vatalinib	65	NR%	6 m = 14%[3]	6 m = 31%[3]	NR
O'Reilly *et al*[69], 2010	Sunitinib	77	1.4%	1.3	3.7	NR
Ko *et al*[67], 2010	Bevacizumab, erlotinib	36	2.8%	1.3	3.4	6 m = 22%[3]
Astsaturov *et al*[100], 2011	Bevacizumab	16	0%	1.4	5.5	NR
Astsaturov *et al*[100], 2011	Bevacizumab, docetaxel	16	0%	1.6	4.2	NR
Milella *et al*[73], 2004	Celecoxib, 5-FU	17	12%	1.9	3.5	NR
Pino *et al*[74], 2009	Celecoxib, capecitabine[2]	35	8.6%	4.0	4.4	NR
Starling *et al*[101], 2012	Imatinib, gem, oxaliplatin	27	7.4%	4.6	5.6	28%
Carvajal *et al*[102], 2009	Flavopiridol, docetaxel	10	0%	1.9	4.2	0%
Nallapareddy *et al*[103], 2010	Sarcatinib	19	0%	1.6	2.5	NR

[1]Intent-to-treat analysis; [2]Pooled analysis of pancreatic (86%) and biliary (14%) cancer; [3]6 m: 6 mo survival rate. 5-FU: 5-Fluorouracil; Gem: Gemcitabine; NR: Not reported; PFS: Progression free survival; OS: Overall survival; TTP: Time to progression.

Among other effects, the EGFR/PI3K/AKT signaling cascade results in activation of the mammalian target of rapamycin (mTOR) protein kinase. mTOR plays a central role in cell growth and cell-cycle control, integrating mitogenic signals from various extracellular ligands including EGF, insulin, and insulin-like growth factor (IGF-1/2). Wolpin *et al*[62] tested the direct mTOR inhibitor everolimus in gemcitabine-resistant disease, but observed no objective responses and a disease control rate of only 21%, with a median PFS of 1.8 mo. A trial of sirolimus monotherapy, in which 75% of patients had received prior chemotherapy, similarly revealed minimal to no clinical activity[63]. Javle *et al*[64] tested a dual inhibition strategy of everolimus in combination with erlotinib in a small phase Ⅱ study, but this study was closed early due to futility.

In a separate (but not unrelated) category, anti-angiogenic strategies, primarily targeting vascular endothelial growth factor (VEGF) and its corresponding receptor (VEGFR), have been extensively studied in pancreatic cancer in both the front-line and salvage settings. The anti-VEGF monoclonal antibody bevacizumab, which did not improve survival when added to either gemcitabine[65] or erlotinib/gemcitabine[66] as first-line therapy in two large randomized phase Ⅲ studies, has also been explored in the refractory setting, with fairly minimal activity. A phase Ⅱ trial by Ko *et al*[67] examined the combination of bevacizumab and erlotinib in gemcitabine-refractory patients and reported a progression-free survival rate of 1.3 mo, with a median OS of only 3.4 mo. Oral TKIs directed against VEGFR have also been explored, including fairly large single-arm phase Ⅱ studies of vatalinib[68] and sunitinib[69]. Sunitinib, tested in the context of a cooperative group study (CALGB 80603), reported a single objective response amongst 77 patients (1.3%), a disease control rate of 22%, and progression-free and overall survival times of 1.3 and 3.7 mo, respectively. Interestingly, recent evidence suggests that pancreatic cancer, despite VEGF/VEGFR upregulation, is poorly vascularized relative to other tumors[70]. These data may help explain the minimal efficacy of anti-angiogenic therapy in pancreatic cancer.

Several other potential oncogenic pathways have been targeted in the second-line setting. Cyclooxygenase-2 (COX-2) is upregulated in pancreatic cancer[71], and its product prostaglandin-E can transactivate EGFR and promote tumor survival[72]. Celecoxib, a selective COX-2 inhibitor, has been tested in combination with fluoropyrimidines (5-FU or capecitabine) in second-line regimens and found to produce response rates of 9%-12% with very mild side effect profiles[73,74]. Ruxolitinib, an oral inhibitor of Janus kinase (JAK) signaling that is approved for use in myelofibrosis, has been evaluated as second-line therapy in combination with capecitabine in a randomized phase Ⅱ trial in patients with refractory pancreatic cancer (NCT01423604); this study has completed accrual as of mid-2013 and results are currently being awaited (Table 4). Data from other studies of targeted therapies are shown in Table 5.

DISCUSSION

There is presently no universally accepted standard of care for patients with advanced pancreatic cancer who have progressed on front-line therapy. As described above, with a few notable exceptions, the vast majority of studies conducted in this setting have been single-arm, single-institution trials with relatively modest sam-

ple sizes. Such non-randomized trials need to be carefully interpreted in light of their inherent selection bias; certainly, those patients who are well enough to consider salvage treatment may already have more favorable tumor biology that influences patient outcomes, including survival rates, independent of the specific choice of therapy.

This argument certainly lends itself in support of randomized phase Ⅱ/Ⅲ trials; studies that fit this category and remain open or are still actively recruiting (as of December 2013) are presented in Table 4. However, it should be recognized that the design and performance of randomized studies in this setting is particularly challenging. As the CONKO investigators observed, a control arm of best supportive care alone, while perhaps appropriate in many cases, is not a particularly attractive option to patients and may hinder study enrollment. But deciding on what the appropriate reference standard should be in a randomized study design, absent compelling evidence to support one regimen over another, is not a straightforward issue. For example, can a fluoropyrimidine alone (capecitabine, S-1, or 5-FU) be considered adequate as a control arm? Some might argue that there are adequate data indicating that a (fluoropyrimidine plus oxaliplatin) combination is clearly superior, and thus represents a more appropriate (and ethical) comparator for a randomized trial. But for a novel agent being evaluated in this setting, does comparing it alone to a reference standard of, for example, FOLFOX, provide adequate study equipoise?

It should also be noted that almost all of the studies detailed above were conducted in the pre-FOLFIRINOX era; as such, they primarily included patients who received a gemcitabine-based regimen as front-line therapy. It would seem logical that for a patient in the present time who receives FOLFIRINOX as first-line therapy, the next step would be to try a gemcitabine-based regimen (monotherapy, gemcitabine/*nab*-paclitaxel, or perhaps another gemcitabine-based combination). However, prospective randomized studies are still required to support this recommendation. Moreover, such FOLFIRINOX-treated patients would obviously not be appropriate for enrollment onto a study in which (s)he might be randomized to receive any of these same drugs, alone or in combination, as part of the control arm. Thus, looking ahead, one must consider the possibility that separate clinical trials should be developed in the second-line setting depending on patients' first-line treatment exposure.

These conundrums highlight only some of the challenges in designing clinical trials in this refractory setting for pancreatic cancer. The other major obstacle hindering progress is the lack of validated predictive biomarkers for this disease that could help inform treatment decisions, whether for conventional cytotoxics or for targeted agents. The track record for targeted agents in chemorefractory pancreatic cancer is particularly dismal, bringing to light the fact that, in the future, we need to be superselective in identifying the patients most likely to benefit from a particular novel therapy, and to develop patient enrichment schemes in clinical trial design accordingly. However, obtaining adequate tumor tissue in this patient population for identifying and validating predictive molecular markers represents a substantial ongoing challenge.

We also propose that certain uniform study benchmarks be established to define "success" for a particular regimen and justify moving on to a larger phase Ⅲ study. A recent systematic review of 34 studies found a median survival for any second line regimen of 6 mo, compared to 2.8 mo for best supportive care alone[75]. With this in mind, thresholds of at least 6 mo for median OS, at a bare minimum, and 4 mo for median PFS, represent reasonable starting points that could be considered clinically meaningful and reflect treatment efficacy that matches or is superior to most historic data reported to date.

Additionally, cost-effectiveness analysis represents an important element to consider embedding within trial design, especially in larger studies, to help inform broader health care decisions in this clinical context in which the magnitude of survival benefit of any novel agent or regimen is likely to be measurable in extra months, if not only weeks. Finally, and perhaps even more importantly, we recommend that every effort should be made to incorporate quality of life (QoL) endpoints/patient-reported outcomes into study design, as these measures are of paramount importance for patients in this late-stage setting.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Pancreatic cancer stroma: Understanding biology leads to new therapeutic strategies

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Abstract

Pancreatic ductal adenocarcinoma (PDA) is among the deadliest cancers in the United States and in the world. Late diagnosis, early metastasis and lack of effective therapy are among the reasons why only 6% of patients diagnosed with PDA survive past 5 years. Despite development of targeted therapy against other cancers, little progression has been made in the treatment of PDA. Therefore, there is an urgent need for the development of new treatments. However, in order to proceed with treatments, the complicated biology of PDA needs to be understood first. Interestingly, majority of the tumor volume is not made of malignant epithelial cells but of stroma. In recent years, it has become evident that there is an important interaction between the stromal compartment and the less prevalent malignant cells, leading to cancer progression. The stroma not only serves as a growth promoting source of signals but it is also a physical barrier to drug delivery. Understanding the tumor-stroma signaling leading to development of desmoplastic reaction and tumor progression can lead to the development of therapies to decrease stromal activity and improve drug delivery. In this review, we focus on how the current understanding of biology of the pancreatic tumor microenvironment can be translated into the development of targeted therapy.

Key words: Pancreatic ductal adenocarcinoma; Stroma; Tumor microenvironment; Pancreatic stellate cells; Cancer associated fibroblast; Sonic hedgehog; Hepatic growth factor; Fibroblast activation protein

Core tip: This is a comprehensive review and an update on recent progresses in understanding the role of tumor microenvironment in the growth, invasion and metastasis of pancreatic cancer. The role of tumor microenvironment in anti-tumor immune response and treatment of pancreatic cancer is also reviewed. How our knowledge in tumor microenvironment is translated into the development of pancreatic cancer therapy is discussed.

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDA) is a devastating disease. It is the 4th leading cause of cancer related deaths in the United States and according to latest statistics the incidence rate is on a rise. The high mortality rates are founded on the fact that PDA is very resistant to chemotherapy and radiation. Most patients are diagnosed at late/metastatic stages of the disease. Less than 20% of patients diagnosed with PDA are eligible for surgical resection, and out of those most present with high incidence of metastasis after resection[1]. This aggressiveness contradicts the finding that majority of the tumor volume is not composed of neoplastic cells, but consists of the stroma/desmoplastic reaction to the cancer[2,3]. In recent years, it has become evident that the desmoplastic reaction is not only a bystander, but it is a source of cellular and molecular components that promote tumor progression and metastasis[4,5]. Importantly, increased levels of stroma correlate with poor prognosis[6] and depletion of the stromal compartment has been associated with improved prognosis in both preclinical and clinical trials[7-9] making pancreatic tumor stroma a valid therapeutic target. Despite the broader understanding of PDA biology very little progress has been made in terms of treatment development. Gemcitabine was approved for PDA treatment over a decade ago, however it still remains the standard of care[10]. The recent breakthrough phase Ⅲ clinical trial evaluating combination therapy FOLFIRINOX (oxaliplatin/irinotecan/5-FU/leucovorin) showed increase in overall survival by 4.3 mo when compared to gemcitabine but it also resulted in increased toxicity. Results of the study led to approval of this drug combination for patients with metastatic PDA and good performance status[11]. The development of new therapeutics for PDA has been progressing very slowly, nevertheless the devastating PDA statistics call for an urgent advance in effective treatment strategy. In this review, we will discuss the current understanding of PDA biology and how this knowledge is being translated into development of novel, targeted therapies for PDA patients.

STROMAL COMPONENTS OF PANCREATIC CANCER

The histological hallmark of PDA is the dense stroma surrounding malignant epithelial cells. The stroma, also referred to as desmoplastic reaction consist of numerous cellular as well as acellular constituents. The cellular components include fibroblasts, stellate cells, immune cells, endothelial cells, and nerve cells. The acellular compartment is comprised of extracellular matrix (ECM) (*i.e.*, collagen, fibrinogen, hyaluronan, and fibrin) as well as variety of other proteins, enzymes, and growth factors (Figure 1).

Fibroblasts

Activated fibroblasts, also referred to as pancreatic stellate cells (PSCs), have been given much attention in the past years. PSCs in their quiescent form are found in minimal numbers in normal, healthy pancreas[12]. Their homeostatic role is still poorly understood, however they have been shown to contain fat droplets in their cytoplasm; indicating potential role in lipid metabolism; have low mitotic index and low capability of ECM synthesis[13]. In PDA, on the other hand, PSCs become activated, as determined by their myofibroblastic phenotype and expression of alpha smooth muscle actin[14]. Activated PSCs have been shown to be a source of ECM, growth factors and immune modulatory signals[15-17]. Molecular signals originating from PSCs are conveyed to neoplastic cell promoting tumor proliferation and invasion, cancer stem cell maintenance and generation of immunosuppressive environment[13,16-21]. Similarly, neoplastic cells send stimulatory signals to PSCs providing a positive feedback loop that promotes cancer progression[22]. The population of stromal fibroblasts is very heterogeneous and numerous markers have been utilized to characterize stromal cells[23]. PSCs, which are regarded as alpha smooth muscle actin expressing cells are phenotypically similar to a broader population of fibroblasts marked by the surface glycoprotein expression of fibroblast activation protein (FAP)[14]. This similarity is based on the ability of both cell types to promote tumor proliferation and invasion, secretion of collagen types Ⅰ, Ⅲ, and Ⅳ, fibronectin, laminin, hyaluronan, and various growth factors[14,24]. Pro-tumorigenic properties of FAP expressing fibroblasts have made them an attractive target for PDA therapy (discussed later).

Extracellular matrix

Another component of the tumor microenvironment is the ECM. This acellular part of pancreatic tumor stroma is composed of variety of fibrous proteins (*i.e.*, collagen), polysaccharides (*i.e.*, hyaluronan) and glycoproteins (*i.e.*, fibronectin). Additionally, diversity of growth factors and other proteins are found in the ECM of PDA. This mesh of fibrous molecules not only provides support to the surrounding tissues but it also plays a role in differentiation, remodeling and homeostasis, in healthy organs[25]. Not surprisingly, different components of pancreatic stroma ECM have been shown to have tumorigenic properties. In particular, collagen I has been associated with higher expression of transgelin (gene used in this study to determine PSCs activation) when compared to other non-activating matrices[26]. In other studies, collagen I was linked to resistance to gemcitabine, a standard cytotoxic drug used for pancreatic cancer treatment[27,28]. Hyaluronan (HA), a non-typical glycosaminoglycan with high capacity of water retention, has recently become an attractive target for pancreatic cancer therapy. HA is expressed in high levels in PDA and its abundance has been connected to increased intratumoral fluid pressure and consequent vascular collapse[29,30]. PDA, unlike many solid tumors is hypovascular, moreover, the blood vessels that are present in the intratumoral space have been reported to be mostly nonfunctional[30]. The lim-

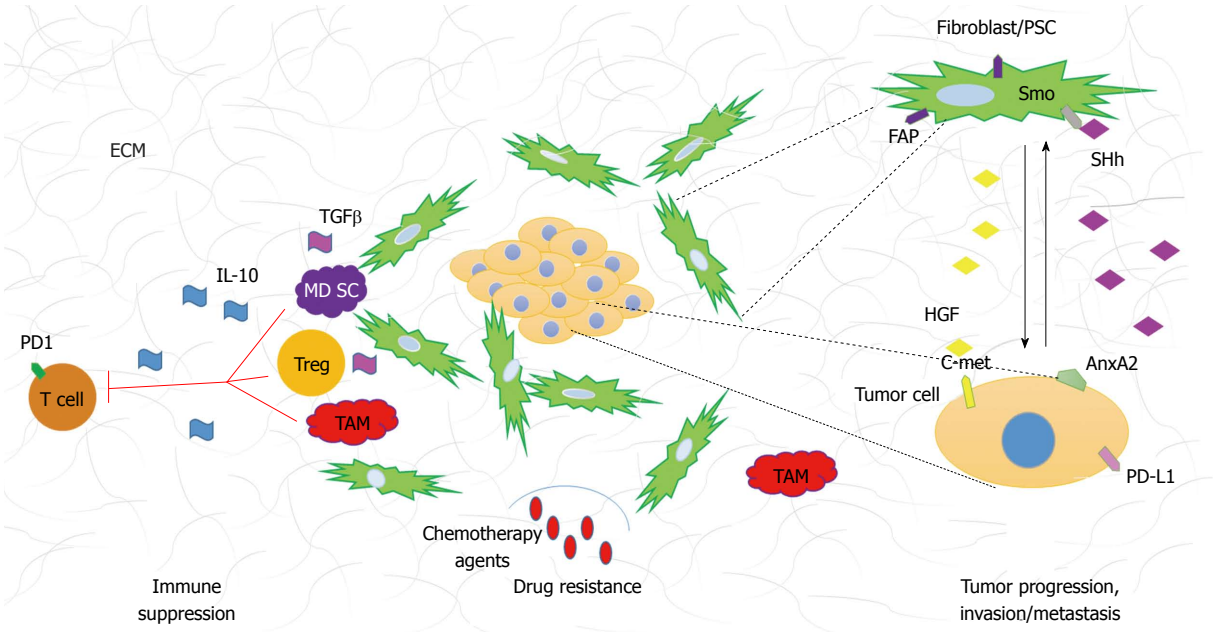

Figure 1 Graphical representation of the stromal components and their interactions in pancreatic ductal adenocarcinoma. FAP: Fibroblast activation protein; Smo: Smoothened; SHh: Sonic hedgehog; HGF: Hepatocyte growth factor; PD-L1: Programmed death ligand-1; PD1: Programmed death-1 (receptor); AnxA2: AnnexinA2; TGFβ: Transforming growth factor β; IL-10: Interleukin 10; MDSC: Myeloid derived suppressor cell; Treg: T regulatory cell; TAM: Tumor associated macrophage.

ited numbers of functional blood vessels in PDA and the dense stroma are believed to be among the reasons why intravenous chemotherapeutic agents as well as the recently tested antiangiogenic drugs[31] do not elicit great effect on the tumor cells.

Immune cells

Broad repertoire of immune cells including both adaptive and innate cell types are also present in the PDA tumor microenvironment. Tumor infiltrating immune cells have been implicated in tumor progression, chemotherapy resistance and metastasis[32-34]. Additionally, the immune infiltration is evident in early premalignant lesions and increases with PDA progression[32]. Immune suppression and immune tolerance to tumor associated antigens is one of the characteristics of PDA and it is associated with poor prognosis[33,35]. The abundance of suppressive cells leads to low numbers of effector $CD8^+$ T cells in the PDA stroma and consequently limited anti-tumor cytotoxicity[32]. Among the most plentiful tumor infiltrating immune cells characterized by their suppressive phenotype are myeloid derived suppressive cells (MDSCs), T regulatory cells (Tregs), and tumor associated macrophages (TAMs). The suppressive cell population is characterized by its ability to prevent activation and functionality of effector cells leading to diminished tumor cytotoxicity[36]. The immune modulatory cell population regulates effector cells anti-tumor responses by variety of mechanisms. MDSC inhibit $CD8^+$ T cell function *via* arginase, and reactive oxygen species secretion, which requires direct cell contact[37,38]. Tregs ability to decrease effectors function is partially due to their ability to secrete suppressive cytokines such as interleukin 10 (IL-10) and tumor growth factor β (TGFβ) but they can also be cell contact dependent where proteins like CTLA-4 and PD-1 are involved[39]. TAMs can be divided into two functional subtypes: M1 (pro-inflammatory) and M2 (immunosuppressive). The M2 subtype cells are a source of anti-inflammatory cytokines such as IL-10 and have been shown to induce Th2 responses (also found to be immunosuppressive in PDA)[40]. In addition to the presence of immunosuppressive cell populations in the PDA microenvironment, the numbers of effector cells such as $CD4^+$, $CD8^+$ T cells and NK cells are minimal. More importantly, infiltrating $CD8^+$ and $CD4^+$ T cells have either naïve phenotype or are nonfunctional, antigen experienced effectors[32]. There are numerous mechanisms that have been implicated in the non-functionality of antigen experienced T cells. Checkpoints and inhibitory receptors like PD-1 are examples of proteins that transduce inhibitory signals during lymphocyte activation[41]. Tumor cells can also express ligands such as the PD-L1 protein that have been shown to dampen immune anti-tumor responses. Upregulation of those inhibitory molecules, PD-L1 in particular, has been associated with poor prognosis[42,43]. Lastly, TGFβ has been shown to play a role in Th17 subtype differentiation[44]. Interestingly, TGFβ dependent differentiation of Th17 cells has been implicated with increased immunosuppressive abilities[45]. Both tumor cells and cancer associated fibroblast secrete TGFβ and increased levels of IL-17 secreting $CD4^+$ cells (Th17) have been found in PDA tumor microenvironment[46]. To date, the role of Th17 subtype of immune cells remains controversial in cancer biology, as it has been shown to have both pro- and anti-tumorigenic properties[47-49]. It is important to mention, that the role of Th17 cells is well documented in promoting fibrosis[50,51]. Hepatic stellate cells (HSCs) have been shown to become activated in

response to Th17 secreted factors[50,52], and because PSCs resemble HSCs, it would be interesting to investigate the role of Th17 immune subtype on PSCs activation and desmoplastic reaction.

Subsequently, modulation of the pro-tumorigenic immune infiltration by either depletion of suppressive cells, polarization of the cell population to more anti-tumor phenotype, checkpoint blockade or increase of activity of the effector cells can be exploited in cancer therapy.

SIGNALING NETWORKS IN THE TUMOR MICROENVIRONMENT

Tumor-stroma interactions create a very complicated signaling network that drives tumor progression. Many signaling pathways have been associated with PDA tumorigenesis, in this review we will focus on paracrine pathways that originate in the neoplasm and contribute to the development of desmoplastic reaction (Figure 1).

Sonic hedgehog

Sonic hedgehog (SHh) is a developmental signaling pathway that is crucial for organ development during embryogenesis. Briefly, in the absence of ligand (SHh) the signaling pathway is inactive and the cell surface receptor Patched (Ptch) inhibits translocation of smoothened (Smo) to the cell surface. Upon ligand binding, Ptch relives the repression on Smo allowing it to translocate to cell surface. The translocation of Smo is a key activating step in downstream signaling. Gli1/2/3, which belong to the zinc-finger transcription factor family are the downstream effectors of Smo activation. Ligand binding to Ptch, results in the translocation of Gli1 (activator) to the nucleus allowing expression of SHh associated genes. During activation of the pathway, Gli2/3 (repressors) are nonfunctional. In the absence of ligand binding, Gli2/3, undergo proteolytic cleavage, move to nucleus and repress transcription of SHh dependent genes. In the inactive state, Gli1 is rendered nonfunctional[53]. SHh is overexpressed by PDA tumor cells, however its function is restricted to the stromal compartment forming a paracrine signaling network that promotes and maintains desmoplasia[54-56]. It has been also noted that only cancer associated fibroblasts and not the neoplastic cells show SHh pathway activation and Smo receptor overexpression[57]. Importantly, Olive *et al*[7] demonstrated that the use of a SHh inhibitor in preclinical mouse model of pancreatic cancer, resulted in better delivery of gemcitabine through reduction of stroma and increase of vascular density. It is important to mention that even though cancer associated fibroblasts (CAFs) are an established target of SHh pathway activation, recent study of pancreatic cancer stem cells (cancer initiating cells) showed that Smo is overexpressed in this population of tumor cells. Pancreatic cancer stem cells alike CAFs have been shown to be susceptible to SHh inhibition and should be considered a target[58].

TGFβ

The notion that depletion of stromal compartment allows for better drug delivery in PDA brought upon re-examination of another signaling pathway that has been linked to regulation of desmoplastic reaction, the TGFβ signaling pathway. This signaling cascade involves three TGFβ ligands and three receptors. In short, binding of ligand to its receptor (type Ⅱ) results in recruitment and phosphorylation of type I receptor and downstream propagation of molecular signals. The effector molecules in this cascade are the proteins of SMAD family, which upon phosphorylation, dimerize, translocate to the nucleus and regulate expression of TGFβ associated genes[59]. TGFβ is overexpressed in PDA and its overexpression correlates with poor survival[60]. TGFβ's involvement in pancreatic cancer is complicated as it has been shown to affect both the stromal and the neoplastic compartments. Elevated levels of TGFβ have been shown to impact cell proliferation, immunosuppression and activation of PSCs[61-64]. In mouse models, overexpression of SMAD 7 (TGFβ inactivator) showed decreased ECM production, less fibrosis and more importantly diminished PSCs activation[65]. Additionally, TGFβ has been demonstrated to drive epithelial to mesenchymal transition (EMT) process, believed to be the initial step of metastasis[66]. EMT was first characterized in development, in which the process is vital for embryogenesis and organogenesis. The cellular characteristics of EMT include the loss of epithelial cells polarity, cell adhesion, gain of mobility and invasive properties resulting in phenotypical changes that resemble mesenchymal cells[67]. On a molecular level, EMT is described by the changes in gene/protein expression that occur in this process. Specifically, upregulation of mesenchymal markers (vimentin, fibronectin, N-cadherin, Snail and Slug) and downregulation of epithelial markers (E-cadherin, zonula-occludens and nuclear translocation of β-catenin) are routinely used to determine the presence of EMT[67,68]. In recent years, many different pathways have been implicated in the initiation of EMT and consequent cancer invasion and metastasis, of which TGFβ is an example[66,69-74]. EMT has also been linked to induction and maintenance of cancer stem cell population in PDA[75]. Importantly, the presence of EMT markers (as discussed above) has been shown to correlate with higher lymph node metastasis and decreased survival in PDA patients[76]. Taken together, the pleotropic functionality of TGFβ in cancer makes it a valid target for patients with PDA.

Others

There are many other signaling pathways that have been associated with PDA development, progression and metastasis. We will discuss them briefly. Expression of Delta-like ligand 4, a protein involved in the developmental Notch pathway has been linked to worst prognosis in patients who underwent surgical resection of pancreatic tumor[77]. Moreover, inhibition of γ-secretase, a protein

that allows Notch signaling propagation to take place and that is often constitutively active in PDA, showed regression of primary tumors, reduced metastasis and decrease of pancreatic stem cell population when combined with gemcitabine[78]. Another pathway that recently gained attention is the c-met pathway. It is well documented that c-met receptor and its ligand HGF are upregulated in PDA. C-met and HGF are detected early in PDA development but are not sufficient to promote tumorigenesis without other oncogenic changes[79]. Recently the expression of c-met has been linked to the stem cell population and because HGF is exclusively secreted by stromal fibroblasts, paracrine relationship between the stroma and neoplasm to promote cancer progression has been suggested. Importantly, studies with c-met inhibitors showed increase of apoptosis and sensitivity to gemcitabine in malignant cells[80]. Moreover, stromal expression of HGF was correlated with decreased disease free survival[81] proposing that HGF/c-met targeting can be beneficial to patients with PDA. Another attractive target is the annexinA2 pathway shown to play an important role in pancreatic tumor metastasis and EMT. Inhibition of tyrosine 23 phosphorylation of annexinA2 was shown to reduce invasion *in vitro,* and metastasis *in vivo*. Although, the kinases responsible for annexinA2 phosphorylation in PDA remain to be confirmed, IGF-1R and Src have been proposed to be involved[74].

Nuclear factor kappa-B (NF-κB), a transcription factor, regulates genes involved in inflammation, cell proliferation and survival[82]. NF-κB signaling pathway activity has been documented to be upregulated in PDA but not in normal pancreatic tissue[83,84]. Activation of this signaling cascade has also been linked to early processes in PDA development. Liou *et al*[85], recently reported that macrophage secreted cytokines initiate acinar to ductal metaplasia *via* activation of NF-κB and consequent upregulation of matrix metalloprotinases (MMPs). To date, direct targeting of NF-κB has been shown to be challenging[86,87]. As an alternative to the direct NF-κB inhibition, upstream activators and downstream effectors of the signaling pathway should be evaluated.

Numerous signaling pathways have been explored as potential targets for pancreatic cancer therapy, however review of all of them goes beyond the scope of this article.

TARGETING OF STROMAL COMPARTMENTS AND CLINICAL APPLICATIONS

Understanding complex stromal constituents and involvement of numerous signaling pathways in PDA progression and desmoplastic reaction is crucial to the development of novel therapies. It has become evident that targeting the stromal components has undeniable benefit for preclinical mouse models of PDA. However, translating those findings to the patients care can be challenging. There are numerous ongoing clinical trials utilizing the above described targets that show encouraging results. In this part of the review we will briefly discuss the most promising ones (Table 1).

SHh

SHh pathway inhibition shows beneficial effect in patients with other cancers such as basal cell carcinoma for which Vismodegib (GDC-0449) has been FDA approved in 2012[88]. Inhibition of SHh in preclinical mouse models showed better gemcitabine delivery, stromal depletion and increased vascularization of PDA tumors[7]. Thus, different SHh inhibitors have recently been tested in clinical trials in combination with gemcitabine or FOLFIRINOX for metastatic PDAs[89]. Additionally, GDC-0449 is now being tested in combination with *nab*-paclitaxel (human-albumin-bound paclitaxel, Abraxane) and gemcitabine in phase Ⅱ clinical trial in patients with previously untreated metastatic PDA (clinical trial # NCT01088815) to evaluate disease free survival and toxicity. Although IPI-926 (Smo inhibitor) given in combination with gemcitabine showed partial responses in 3 out of 9 patients, the combination of IPI-926 and gemcitabine did not yield any survival benefit comparing to gemcitabine alone[90]. Therefore, targeting the stroma of PDA through SHh inhibition and simultaneous modulation of other stromal signaling should be explored.

Hyaluronidase

Another stromal target showing encouraging results in phase Ib clinical trials is hyaluronan. As shown in mouse models of PDA, enzymatic degradation of hyaluronan resulted in increased gemcitabine tumor cytotoxicity due to relief of vascular collapse[30]. Those prove of principle experiments lead to the development of PEGPH20 (pegylated recombinant human hyaluronidase- an enzyme that degrades hyaluronan). Administration of PEGPH20 to PDA patients with advanced disease (stage Ⅳ) in combination with gemcitabine revealed partial response in 43% of patients and stable disease in additional 30% patients in phase Ⅰb clinical trials. More impressively, the partial response rate was 64% in those patients whose PDAs expressed high level of hyaluronan[91]. This high response rate has led to further testing of PEGPH20 in combination with gemcitabine and *nab*-paclitaxel in a randomized phase Ⅱ clinical trial.

TGFβ

Trabedersen, a type Ⅱ TGFβ antisense inhibitor is also being tested in clinical trials in patients with advanced pancreatic adenocarcinoma and malignant melanoma. Although phase Ⅱ clinical trial results have not been released yet, phase Ⅰ reports revealed that trabedersen was well tolerated and showed median overall survival to be 13.2 mo. In addition, one patient presented with a stable disease after 14.8 mo of last treatment[92].

Immune system modulation/activation

Immunotherapy approaches are being tested in clinical

Table 1 Recent and ongoing preclinical and clinical studies of experimental therapies targeting tumor microenvironment of pancreatic ductal adenocarcinoma

Stromal component	Therapeutic target	Treatments in preclinical and clinical trials	Up to date preclinical/clinical trial results
PSCs/fibroblasts	FAP	Sibrotuzumab (colorectal cancer)	Hofheinz *et al*[99], 2003
ECM	Hyaluronan	PEGPH20	Strimpakos *et al*[91], 2013
	MMPs	BAY 12-9566	Moore *et al*[100], 2003
		Marimastat	Bramhall *et al*[101], 2002
Immune cells	PD-L1	BMS-936559	Brahmer *et al*[102], 2012
	CTLA-4	Ipilimumab	Le *et al*[95], 2013
	$CD8^+$ T cells	GVAX	Lutz *et al*[93], 2011
			Laheru *et al*[94], 2008
	CD40	CP-870,893	Beatty *et al*[103], 2013
Signaling pathways mediating tumor-stroma interactions	Smo/SHh	Vismodegib (GDC-0449)	Stephenson *et al*[90], 2011
		IPI-926	
	Type Ⅱ TGFβ receptor	Trabedersen	Oettle *et al*[92], 2009
	γ-secretase (Notch pathway)	PF-03084014 (preclinical)	Yabuuchi *et al*[78], 2013 (preclinical)
	HGF/c-met	Many different compounds (solid cancers)	Venepalli *et al*[104], 2013 (solid cancers)
	Different molecules in NF-κB cascade	Many different compounds (*i.e.*, curcumin, proteasome inhibitor)	Arlt *et al*[105], 2012

ECM: Extracellular matrix; MMP: Matrix metalloproteinase; PD-L1: Programmed death receptor ligand 1; CTLA-4: Cytotoxic T-lymphocyte antigen 4; SHh: Sonic hedgehog; Smo: Smoothened; TGFβ: Transforming growth factor β; HGF: Hepatocyte growth factor; NF-κB: Nuclear factor κ-B; PSC: Pancreatic stellate cell; FAP: Fibroblast activation protein.

trials for PDA with a goal to induce tumor infiltration and activation of effector cells (*i.e.*, $CD8^+$ T cells) and consequent $CD8^+$ T cell dependent tumor lysis.

Multiple clinical trials of a lethally irradiated allogeneic GM-CSF secreting whole cell vaccine (GVAX) administered to patients with resected PDA or metastatic PDA demonstrated that enhanced response of interferon-γ secreting mesothelin-specific $CD8^+$ T cells in peripheral lymphocytes correlates with better survival[93,94]. A pilot study testing the combination of GVAX and ipilimumab (an anti-CTLA-4 therapeutic antibody) comparing to ipilimumab alone showed a trend of increase in overall survival in metastatic PDA patients that have been previously treated with multiple lines of chemotherapy and thus supported the role of CTLA-4 blockade in enhancing anti-tumor response of GVAX[95]. However, it remains to be explored how vaccine-based immunotherapy activates anti-tumor effector cells within tumor microenvironment. Identification of new targets in tumor microenvironment may enhance the development of immune modulatory therapies.

A potential immune modulatory target in tumor microenvironment is CD40. CD40 is a costimulatory molecule found on antigen presenting cells (APCs) that is required for their activation by $CD4^+$ helper cells. Only activated APCs can in turn activate naïve $CD8^+$ T cells into cytotoxic effector cells. Key studies showed that using CD40 activating antibody can effectively stimulate APCs in the absence of $CD4^+$ helper cells, which then can successfully prime and activate $CD8^+$ T cells[96]. Those preclinical studies led to development of activating CD40 antibodies, which have been tested in clinical trials. One study showed that combination of CD40 agonist with gemcitabine resulted in tumor regression in patients not eligible for tumor resection. Interestingly, it was noted that the tumoricidal cells were CD40 activated macrophages and not $CD8^+$ T cells as originally expected. The treatment with CD40 agonist resulted in stroma depletion and increased numbers of tumor infiltrating activated macrophages[8].

How stromal fibroblast cells can modulate anti-tumor immune response has been investigated in preclinical studies. One study demonstrated that depletion of fibroblast activation protein-α (FAP)-expressing stromal cells in PDA resulted in an immune-mediated hypoxic necrosis of both tumor and stroma cells[97]. Additionally, targeting of cancer stroma fibroblasts with FAP-activated promelittin protoxin, showed increased tumor lysis and growth inhibition in xenograft mouse models of breast and prostate cancer[98]. However, targeting FAP positive stromal cells with humanized anti-FAP antibodies tested in phase Ⅱ clinical trials in patients with metastatic colorectal cancer did not report encouraging results[99]. Taking into consideration the outcomes from both preclinical and clinical studies, it is reasonable to propose that FAP-targeted stromal depletion shows immune activating effect, but requires additional immune modulation to be effective. It is plausible that simultaneous FAP-targeted stromal depletion and immune activation, by either vaccination or immune checkpoint blockade would result in increased benefits for PDA patients.

CONCLUSION

Despite the broad number of clinical trials, there is still a lack of groundbreaking therapies for patients affected by pancreatic cancer. Thus, targeting only the neoplastic cells has not resulted in a substantially improved PDA

treatment. It is now well established that the desmoplastic reaction present in PDA is not just a bystander but it is a source of different cellular and acellular factors that promote tumor progression, immunosuppression and metastasis. Targeted therapies to deplete stromal compartments have shown improved chemotherapy delivery and reduction of immunosuppression in preclinical models. There is still much work to be done in order to decipher the complicated interactions between stroma and neoplastic cells in PDA. It is clear, however, that future studies should not be limited to one component of PDA. Application of targeted therapy to deplete the tumorigenic stromal compartment along with inhibition of cancer promoting signaling pathways should be evaluated. Moreover, future studies ought to test the combination of agents that target the stroma and those that activate anti-tumor immune responses. Treatments that can reduce desmoplastic reaction, overcome immune suppression and inhibit tumorigenic signaling pathways may lead to more successful patient care.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Embryonic stem cell factors and pancreatic cancer

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Abstract

Pancreatic ductal adenocarcinoma (PDAC), the most common type of pancreatic tumor, is a highly aggressive human cancer with the lowest five-year survival rate of any human maligancy primarily due to its early-metastasis and lack of response to chemotherapy and radiation. Recent research suggests that PDAC cells comprise a hierarchy of tumor cells that develop around a population of cancer stem cells (CSCs), a small and distinct population of cancer cells that mediates tumoregenesis, metastasis and resistance to standard treatments. Thus, CSCs could be a target for more effective treatment options. Interestingly, pancreatic CSCs are subject to regulation by some of key embryonic stem cell (ESC) transctiption factors abberently expressed in PDAC, such as SOX2, OCT4 and NANOG. ESC transcription factors are important DNA-binding proteins present in both embryonic and adult somatic cells. The critical role of these factors in reprogramming processes makes them essential not only for embryonic development but also tumorigenesis. Here we provide an overview of stem cell transcription factors, particularly SOX2, OCT4, and NANOG, on their expression and function in pancreatic cancer. In contrast to embryonic stem cells, in which OCT4 and SOX2 are tightly regulated and physically interact to regulate a wide spectrum of target genes, *de novo* SOX2 expression alone in pancreatic cancer cells is sufficient to promote self-renewal, de-differentiation and imparting stemness characteristics *via* impacting specific cell cycle regulatory genes and epithelial-mesnechymal transtion driver genes. Thus, targeting ESC factors, particularly SOX2, could be a worthy strategy for pancreatic cancer therapy.

Key words: Embryonic stem cells; NANOG; SOX2; OCT4; Pluripotency; Pancreatic cancer; Cancer stem cells

Core tip: Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal human cancer due to its early metastasis and lack of response to chemoradiotherapy. Pancreatic cancer stem cells (CSCs) are implicated in tumorigenesis and metastasis as well as therapy resistance, therefore represent a potential target for effective therapeutic options. Recent publications including our own research demonstrate that key embryonic stem cell (ESC) factors, such as OCT4, NANOG and SOX2, are abbrently expressed in PDAC and contribute to pancreatic CSC-like characteristics, such as self-renewal and de-differentiation. This review aims to summarize our current knowledge on the role of ESC

factors particulary SOX2 in regulating pancreatic CSC-like feature and implication for therapy.

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INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer death in men and women in the United States. In 2012 alone, an estimated 43920 adults in the United States were diagnosed with pancreatic cancer and 37390 deaths from this disease ocurred[1]. About 280000 new cases of pancreatic cancer were recorded in 2008 worldwide. Pancreatic ductal adenocarcinoma (PDAC), the most common type of pancreatic cancer, is also the most lethal among the human solid tumors with a 5-year survival rate of less than 5 percent[2]. The main reasons for this outcome include lack of early detection, invasive behavior and intrinsic resistance to most chemo-/radio- and immuno-therapy strategies[3,4]. Recently, several studies have identified PDAC cancer stem cells (CSCs), which are highly tumorigenic and have the capacity to not only self-renew, but also generate differentiated progeny[3,5-7]. Pancreatic CSCs are also resistant to chemotherapies commonly used to treat patients with PDAC[8-10]. Thus, studies identifying key determinants in pancreatic cancer and pancreatic CSCs can provide both biomarkers of PDAC aggressiveness and potentially optimal targets to overcome chemoresistance (Table 1). Here we review how embryonic stem factors contributes to the agressiveness of this disease and the potential for targeted therapy.

STEM CELL DEFINITION AND TYPES

Stem cells (SCs) are traditionally defined as cells that can both self-renew and generate a progeny that are capable of following more than a single differentiation pathway[11]. Currently, four types of SCs have been described[12]. The first two are physiologically present at different stages of life, namely, the embryonic stem cells (ESCs) and the somatic or adult stem cells (ASCs). The ESCs are the best studied SCs and knowledge derived from ESCs research has guided the investigations of other types of SCs. ASCs are postnatal derivatives of ESCs located throughout the body. ASCs have been shown to retain co-expression of at least three of the core transcription factors characteristic of ESCs (OCT4, KLF4, and SOX2). Similar to ESCs, the presence of a balanced network of core stem markers, rather than the overt expression of a single factor, contributes to maintainenance of ASC characteristics. The third SC type is induced pluripotent stem cells (iPSCs), which are artificially engineered from a non-pluripotent cell, such as *via* somatic cell nuclear transfer or reprograming with gene transfer. The generation of iPSCs represents a milestone achievement in SC research, which not only breaks the dogma that somatic cell differentiation is an irreversible process, but also makes possible a new approach for regenerative medicine without controversial use of embryos. The fourth SC type is CSCs, also referred to as cancer initiating cells (CICs), which are defined as those cells within a tumor that can self-renew, produce differentiated progeny, and drive tumorigenesis. The ability of cancer cells to form nonadherent spheroids *in vitro* culture is frequently used as a surrogate of stemness. Unlike ESCs, CSCs are highly heterogenous with great variation among the markers for each tumor type.

ESCs AND ESC TRANSCRIPTION FACTORS

ESCs are derived from the inner cell mass (ICM) of the preimplantation mammalian embryo and can be maintained indefinitely in culture[13]. By definition, ESCs are pluripotent. They are able to give rise to all somatic and the three germ cell lineages of the developing embryo. Pluripotency is maintained through self-renewal, which allows ESCs to duplicate themselves without losing the ability to differentiate. This can be achieved *via* both symmetric and asymmetric cell divisions[14].

Over the last decade, there has been accumulating evidence indicating that the maintenance of pluripotency in ESCs is governed by core genetic and epigenetic regulators, which allow self-renewal and prevents specific differentiation pathways. Recent progress on the molecular mechanism(s) governing stem cells pluripotency has provided critical insights into the role of nine core transcription factors OCT4 (POU5F1), NANOG, SOX2, Dppa4, Dppa5, Sall4, Utf1, Rex2, and Rif1 in maintaining mouse cells in the undifferentiated stage[15-18]. Among these genes, OCT4, NANOG, and SOX2, referred to as pluripotency genes, are highly expressed in the ICM. The perfect balance of these proteins maintains pluripotency and self-renew in ESC during the first days of embryonic development[18]. Broadly, the pluripotency genes have been shown to be common to all SC types (Figure 1). In contrast to *OCT4*, *NANOG* and *SOX2*, *c-MYC*, an important oncogene as well as a reprogramming factor for pluripotency[17], is highly heterogeneous in cells from the ICM. However, it is not always considered a pluripotency gene in ESCs. The activity of these three core pluripotency genes regulates and coordinates the expression of a second set of core genes, which include transcription factos, cell surface markers, ABC transporters, and enzymes. Together, these proteins orchestrate the specific stem cells properties[19].

SOX2 and OCT4 form a protein complex in the nucleus of ESCs. This complex is auto-regulated in a loop

Table 1 Biological role and clinical implications for embryonic stem cell factors in pancreatic cancer

Gene	Biological role/behaviour	PDAC implications	Ref.
OCT4	Overexpressed in 69% of PDAC. Pro-oncogenic role	Correlation with N1/M1 status and indicative of worse prognosis	Polvani *et al*[47], 2013
	Overexpressed in 48.8% of PDAC. Induces cell proliferation, migration and invasion	Contribution to metastasis and drug resistance	Lu *et al*[50], 2013
	Overexpressed in human cell lines	Multidrug resistance and metastasis	Wang *et al*[52], 2013
	Induction of tumorigenic capacity	Chemo-resistance	Wang *et al*[53], 2013
	Overexpressed in 79.2% metaplastic ducts	Early carcinogenesis and worse prognosis	Wen *et al*[49], 2010
SOX2	Overexpressed in poorly differentiated human tumors	Correlation to aggressiveness	Sanada *et al*[54], 2006
	Ectopic expression in 19.3% of PDAC. Promotes cancer cell proliferation/dedifferentiation	Rapid tumor progression and poor differentiation	Herreros-Villanueva *et al*[38], 2013
	Induction of tumorigenic capacity	Chemo-resistance	Wang *et al*[53], 2013
NANOG	Overexpressed in 53.5% of PDAC. Induces proliferation, migration and invasion	Associated with eraly stage carcinogenesis and worse overall survival	Lu *et al*[50], 2013
	Overexpressed in cells capable of initiating spheres	Resistance to 5-FU treatment	Lonardo *et al*[68], 2013
	Overexpressed in pancreatic tumors	Contribution to carcinogenesis and correlates to worse prognosis	Wen *et al*[49], 2010

PDAC: Pancreatic ductal adenocarcinoma.

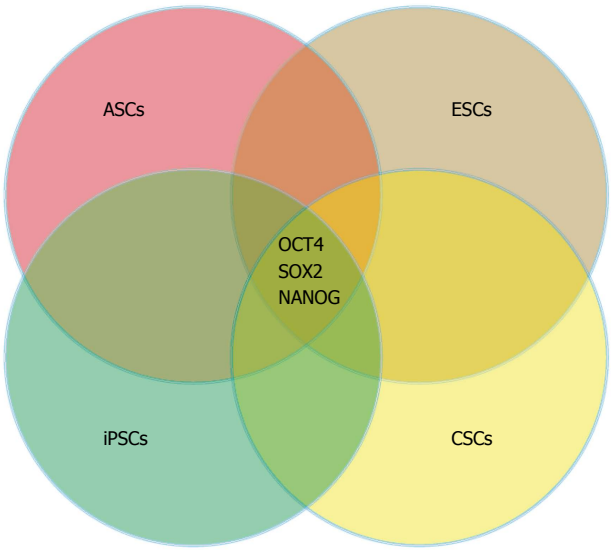

Figure 1 Overlapping expression of SOX2, NANOG, and OCT4 in all four types of stem cells: embryonic stem cells, adult stem cells, induced pluripotent stem cells, and cancer stem cells. ASCs: Adult stem cells; ESCs: Embryonic stem cells; iPSCs: Induced pluripotent stem cells; CSCs: Cancer stem cells.

that, transcriptionally, also induces the expresion of pluripotency genes (most importantly NANOG), cell cycle, apoptosis, DNA repair, chromatin structure genes, and genes regulating endoderm, mesoderm, and ectoderm differentiation. Thus, tight control of all these genes may allow ICM cells to exit from their inherent developmental program, as they acquire the ability to self-renew, while retaining pluripotency as ESCs[20]. Finally, when the expression of these pluripotency genes decreases in a properly regulated way, an induction in the expression of early differentiation markers occurs. These markers include ectoderm markers (*Pax6*, *Otx1*, *Neurod1*, *Nes*, *Lhx5*, and *Hoxb1*), mesoderm markers (*Tbx2*, *T*, *Nkx2-5*, *Myod1*, *Myf5*, *Mesdc1*, *Mesdc2*, *Kdr*, *Isl1*, *Hand1* and *Eomes*), endoderm markers (*Onecut1*, *Gata4*, *Gata5*, and *Gata6*), and extraembryonic markers (*Cdx2* and *Tpbpa*).

KEY ESC FACTORS IN IPSCs

iPSCs were first derived by the transduction of mouse and human fibroblasts through integrating viruses carrying four transcription factors: OCT4, SOX2, MYC and Krupple-like factor 4 (KLF4)[21], also referred as the Yamanaka factors. Takhashi and Yamanaka[21] broke a dogma in developmental biology by showing that mammalian somatic cell differentiation is a reversible process[17,21]. By transfecting human somatic cells with the four Yamanaka factors, they were able to revert the differentiated cells to an embryonic-like state. Because these newly generated cells showed the morphology, pluripotency, and capacity to form teratomas similar to ESCs, they named these cells iPSCs. Later, Yu *et al*[22] further demonstrated that the combination of OCT4, NANOG, SOX2 and Lin28, also called Thomson Factors, was able to produce iPSCs. Both, Yamanaka and Thomson Factors are Reprogramming Factors as Reprogramming is the process that converts differentiated cells back to pluripotent cells, namely the reversal of differentiation.

Recently, new methods have been developed to reprogram human somatic cells with or without MYC[22,23] and to combine only some of the reprogramming transcription factors with chemical inhibitors[24-26]. However, the fact remains that OCT4, SOX2, MYC, and KLF4 reside at the heart of the reprogramming process. Given that the transcription factors in this network not only associate with one another, but also associate with many of the same proteins in the network, there is a high degree of interdependence between these transcription factors. Thus, it is not surprising that the levels of SOX2 and OCT4 need to be controlled carefully for optimal production of iPSC, or that small changes in the levels of these master regulators can lead to dramatically altered cell fates. However, it remains to be determined how

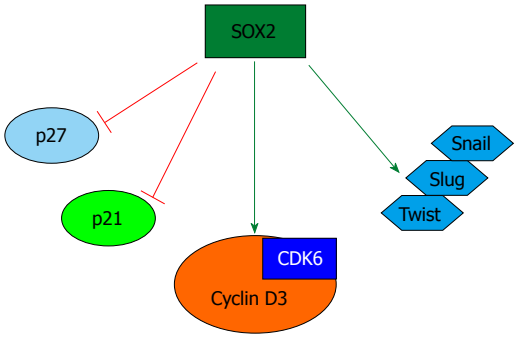

Figure 2 Diagram depicting the molecular mechanism underlying SOX2 expression-induced self-renewal and pluripotency in pancreatic cancer stem cells.

their levels affect the molecular efficiency of reprogramming. Given the strict requirement for SOX2 and OCT4 during development, their key roles in ESC differentiation, and the pronounced differences in reprogramming when their levels are not optimized, additional efforts should be made to determine why small changes in the levels of these two master regulators alters the behavior of pluripotent stem cells.

ESC FACTORS AND CSCs

Although initially discovered in hematopoietic malignancies, such as acute myelogenous leukemia and chronic myelogenous leukemia[27,28], CSCs were later described in various solid tumors, including glioblastoma[29], melanom[30], prostate[31], colon[32] head and neck squamous cell carcinoma[33], breast[34], ovarian[35], bladder[36], lung[37] and pancreatic cancer[6,7,38,39]. In these malignancies, a small population of CSCs can self-renew and differentiate into all of the other cell types forming the bulk tumoral population. However, the bulk of tumor cells lack the ability to differentiate into other subpopulations of cancer cells and thus possess limited self-renewal capacity. In addition, it has been shown that CSCs have tumor initiation capacity, forming xenograph tumors in mice and are radio- and chemo-resistant, contributing to lack of therapeutic response in patients[39].

Although several proteins have been proposed as CSC markers, there is great variation between tumor types[40,41]. This variation might be the result of the lack of standardized techniques to obtain and analyze CSCs, as well as the intrinsic plasticity of these cells[40]. Since CSCs express many genes in common with early ESCs, primarily OCT4, NANOG, and SOX2, the picture that emerged was that these transcription factors could also work together as part of a highly integrated network to regulate pluripotency and self-renewal in tumors. Nevertheless, the heterogeneity of tumors and the plasticity that characterize CSCs render the expression pattern of these transcription factors highly heterogeneous in different tumors and even within the same tumor.

Several publications show that overexpression of OCT4, SOX2 and NANOG, together or separately, led to tumor transformation, tumorigenicity, tumor metastasis, and even distant recurrence after chemoradiotherapy[42]. It is well known that these transcription factors are more frequently overexpressed in poorly differentiated tumors (compared to well differentiated tumors) and, in theory, that the expression level of the pluripotent transcription factors should decrease with the differentiation of cells[43]. In this regard, how these genes contribute to specific CSC properties has not been fully elucidated. Based on data obtained from iPSCs, several mechanisms have been proposed to explain the properties that these transcription factors could be imparting on CSCs. For example, once these transcription factors are overexpressed, they might activate several genes whose promoters are accessible to them. These "first responders" must then engage the epigenetic machinery to remodel the chromatin through histone modification and DNA methylation. In this process, genes critical for pluripotency must be switched on, while genes responsible for differentiation must be turned off and kept off[44]. From this data, it is clear that OCT4, NANOG and SOX2 are master regulators, which together drive the transition from a somatic cell to either a CSC or iPSC (Figure 2).

ESC FACTORS AND PANCREATIC CANCER

As mentioned above, CSCs have also been described in PDAC (Table 1). Originally Li *et al*[6] identified human pancreatic CSCs as $CD44^+/CD24^+/ESA^+$. A few months later, Hermann *et al*[7] showed that CD133 and CXCR4 are also expressed in cells with CSC properties. In addition, some other markers such as c-Met[5] and aldehyde dehydrogenase 1 activity (ALDH1)[45] have been demonstrated in pancreatic CSCs. Recently, some reports describe the presence of a side population (SP) of cells in pancreatic cancer, a chemoresistant population of cells that could be enriched in CSCs. Additionally, this data indicates that SP cells express pancreatic CSC markers (*CXCR4*, *CD133*) and multidrug resistance genes (*ABCB1*), associating these cells with candidate therapeutic targets and potential prognostic value[46].

The regulation and characterization of CSCs in various types of human cancer, in which SOX2, OCT4 and NANOG are important players, is currently a hot topic. However, the number of specific publications analyzing their role in pancreatic cancer is very limited. In particular, a literature search on PUBMED database using the terms "OCT4", "NANOG" and "SOX2" together with "pancreatic cancer", showed 24, 27, and 20 published articles, respectively. Furthermore, only a few of these articles discuss these factors in the context of CSCs (Table 1). Polvani *et al*[47] found that OCT4 is expressed in 69% of PDAC and that this expression correlates with N1/M1 status and clinical stage, being an independent prognostic factor for worst outcomes. In agreement with several breast cancer publications[48], patients with $OCT4^+$ PDAC have a shorter survival, suggesting this ESC factor

as a marker of poor prognosis. Importantly, high levels of OCT4 and NANOG in human pancreatic cancer tissues were found to be associated with early stages of carcinogenesis[49] and correlate with worse prognosis[50]. Additionally OCT4 seems to contribute to multidrug-resistance and metastasis[51,52]. Wang *et al*[53] recently demonstrated that SP cells positive for NANOG, OCT4 and SOX2 possessed aggressive growth, invasion, migration and drug-resistance properties.

To date, very little is known regarding how OCT4 and NANOG contribute to pancreatic CSC properties at the molecular level. Interestingly, recent studies suggest that SOX2 is aberrantly expressed in a significant fraction of pancreatic tumors. Initially, Sanada *et al*[54] analyzed 14 cases of human PDAC immunohistochemically, and observed weak expression of SOX2 in pancreatic intraepithelial neoplasia (PanIN-3) lesions. They also observed relatively high and frequent expression in invasive and poorly differentiated PDAC. Later, it was shown that at the mRNA level, SOX2 expression driven by hedgehog-EGFR signaling is necessary for tumor-initiating pancreatic cancer cells[55]. Very recently, the molecular mechanism underlying SOX2 regulation of pancreatic cancer stemness has been elucidated. Using primary human cancer tissues and cell lines (L3.6, Bxpc3, CFPAC-1, Panc1 and Panc04.03), our group demonstrated a critical role for SOX2 in promoting cell proliferation, dedifferentiation and impartment of stem cell-like features to pancreatic cancer cells[38]. In particular, SOX2 gene suppression arrested cells at the G_1 phase and its overexpression alone was sufficient to drive cell proliferation by facilitating G_1/S transition. Mechanistically, G_1 arrest in SOX2 knockdown cells is associated with a marked induction of $p21^{Cip1}$ and $p27^{Kip1}$, two key cyclin/CDK inhibitors, whereas SOX2 overexpression induces G_1/S-specific cyclin D3 expression. All of three cell cycle regulators were identified as *bona fide* SOX2 regulatory targets. SOX2 also confers pancreatic cancer cell stemness and its overexpression alone is sufficient to drive sphere-formation and expression of CSC markers[7,38,45,56], as well as induce EMT drivers such as Snail, Slug and Twist (Figure 2). Consistently, loss of *miR-145* elevates SOX2 and impairs differentiation in pancreatic tumors[57].

It is now evident that the core stem cell factors OCT4[16], SOX2[58], and NANOG[59] play essential roles in the maintenance of pluripotency and self-renewal of ESCs, ASCs, iPSCs and CSCs. These stem cell factors promote self-renewal by interacting with other transcription factors (Stat3, Hesx1, Zic3), critical cell signaling molecules (Hedgehog, TCF3, FGF2, LEFTY2)[60], and have been found aberrantly expressed in several types of human tumors including pancreatic cancer[61-63]. Although ESCs and CSCs share the property of self-renewal, they also reveal distinct features in that ESCs favor differentiation, whereas CSCs are more biased toward proliferation and inhibition of apoptosis. In particular, SOX2 has demonstrated OCT4 and/or NANOG independent activity in pancreatic cancer cells in promoting cell proliferation, survival, and/or de-differentiation[38]. Recent work by Polvani *et al*[47] further supports this statement demonstrating that OCT4 silencing reduces OCT4 and increases NANOG, but does not alter SOX2 expression.

CSCs AS TARGET FOR CANCER THERAPY

SOX2 immunoreactivity has been demonstrated in PanIN lesions, as well as moderately and poorly differentiated tumors, which is consistent with previous reports showing an enrichment of SOX2 in pancreatic CSCs[64], as well as a decreased expression after anti-ESCs therapies[55,65]. Since SOX2 appears to be a key factor aberrantly expressed in PDAC and confers CSCs-like properties[38], targeting SOX2 or its upstream regulator(s) may be exploited for therapeutic purposes. Recent reports demonstrate that using poly (lactide-co-glucolide) to knockdown DCLK1 results in an increase in miR-145 associated with decreased puripotency factors including SOX2, and consequently, tumor growth arrest in xenografts[57,66]. Lastly, data from Sobrevals *et al*[67] elucidates the relevance of uPAR-controlled oncolytic adenoviruses in the elimination of pancreatic CSCs. Along these lines, C-Met inhibitors have been demonstrated to overcome gemcitabine resistance and stem cell signaling through downregulation of CSC markers including SOX2[65]. Strategies to target CSCs for cancer therapy have been proposed and are under investigation. For instance, metformin directed against pancreatic CSC has been shown to reduce tumor burden and prevent disease progression[68]. Disulfiram, an ALDH inhibitor, was tested *in vitro* and *in vivo* demonstrated a capacity to suppress pancreatic CSCs[69]. Promising results suggest that HAb18G/CD47 or Phospho-valproic acid (MDC-1112) could also be a promising target in pancreatic cancer surrogating anti-STAT3 therapies[70,71]. More recently, HAb18G/CD147 has been identified as another promising therapeutic target for highly aggressive pancreatic cancer and a surrogate marker in the STAT3-targeted molecular therapies, such as by phospho-valproic acid (MDC-1112), a novel valproic acid derivative. Since targeting CSCs has been demonstrated to be a viable therapeutic strategy against pancreatic cancer, a better undertanding of OCT4, NANOG and particularly SOX2 on their expression and regulatory circuitry in PDAC will facilitate the design of individualized therapies for PDAC patients.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Management of borderline and locally advanced pancreatic cancer: Where do we stand?

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Abstract

Many patients with pancreas cancer present with locally advanced pancreatic cancer (LAPC). The principle tools used for diagnosis and staging of LAPC include endoscopic ultrasound, axial imaging with computed tomography and magnetic resonance imaging, and diagnostic laparoscopy. The definition of resectability has historically been vague, as there is considerable debate and controversy as to the definition of LAPC. For the patient with LAPC, there is some level of involvement of the surrounding vascular structures, which include the superior mesenteric artery, celiac axis, hepatic artery, superior mesenteric vein, or portal vein. When feasible, most surgeons would recommend possible surgical resection for patients with borderline LAPC, with the goal of an R0 resection. For initially unresectable LAPC, neoadjuvant should be strongly considered. Specifically, these patients should be offered neoadjuvant therapy, and the tumor should be assessed for possible response and eventual resection. The efficacy of neoadjuvant therapy with this approach as a bridge to potential curative resection is broad, ranging from 3%-79%. The different modalities of neoadjuvant therapy include single or multi-agent chemotherapy combined with radiation, chemotherapy alone, and chemotherapy followed by chemotherapy with radiation. This review focuses on patients with LAPC and addresses recent advances and controversies in the field.

Key words: Pancreas; Locally advanced; Chemotherapy; Radiation; Irreversible electroporation; Pancreatic cancer

Core tip: While the management of resectable patients is surgery (with or without neoadjuvant therapy), and the management of grossly metastatic patients is palliative with systemic chemotherapy with or without radiation, there is an intermediate subset of patients with locally advanced disease which is less straightforward. This review focuses on this unique population of patients with locally advanced pancreatic adenocarcinoma and addresses recent advances and controversies in this field.

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INTRODUCTION

Pancreatic adenocarcinoma is a lethal disease with a high metastatic potential. In 2012, there were an estimated 43920 patients diagnosed with pancreas cancer, and 37390 were expected to die from their disease[1]. The only available potential cure for pancreas cancer is surgical re-

section, with only 15%-20% of patients presenting with pancreas cancer being candidates for resection. For those patients that go onto resection, the 5-year survival ranges from 15%-20%, whereas the 5-year survival for all pancreas cancer patients combined is 3%[1,2].

The factors that lead to the overall dismal prognosis of pancreatic cancer are multiple and varied, making management a challenge. These factors include absence of nonspecific symptoms that leads to delayed diagnosis, biological aggressiveness which is resistant to chemotherapy, and surgical considerations which can be technically demanding[3,4]. While the management of resectable patients is surgery (with or without neoadjuvant therapy), and the management of grossly metastatic patients is palliative with systemic chemotherapy with or without radiation, there is an intermediate subset of patients with locally advanced disease which is less straightforward. This review focuses on this unique population of patients with locally advanced pancreatic adenocarcinoma and addresses recent advances and controversies in this field.

DIAGNOSIS OF LOCALLY ADVANCED PANCREAS CANCER

As technology has evolved, the tools available to evaluate locally advanced pancreas cancer (LAPC) have become more accurate. The principle tools used for diagnosis and staging of LAPC include endoscopic ultrasound (EUS), axial imaging with computed tomography (CT) and magnetic resonance imaging (MRI), and diagnostic laparoscopy[5]. Endoscopic ultrasound provides images of the pancreas and surrounding vessels, and in particular allows for tissue diagnosis with the capability to biopsy. Endoscopic retrograde cholangiopancreatography (ERCP) can be performed at the same time if there is an indication to stent the common bile duct. Therefore, EUS can diagnose the tumor with biopsy, stage the tumor by size and vascular involvement, and use ERCP to therapeutically stent the common bile duct, should it be necessary.

CT with intravenous contrast provides multiplanar, high-resolution, three-dimensional images of the pancreatic tumor, its surrounding vascular structures, and possible lymphadenopathy and liver metastases. Warshaw *et al*[6] demonstrated that more than 90% of patients deemed unresectable by CT are actually unresectable at operation. MRI can also be used to assess extent of tumor involvement and has shown to be equivalent to CT[7]. Difficulties with CT and MRI include measuring response to treatment, particularly in patients who have undergone treatment with radiation therapy[8]. However, with developments in imaging technology, assessment of staging and tumor response is likely to only improve for the patient with pancreatic cancer.

Another pitfall for current axial imaging is the limitation to incompletely visualize potentially small (1-2 mm) tumor deposits[9]. This is critical to the management of pancreas cancer, as patients with extra-pancreatic disease have the same dismal prognosis as those with metastatic disease, and these patients should not be put at risk from a potentially morbid laparotomy or pancreatectomy. This problem can be addressed using diagnostic laparoscopy to directly visualize the intra-abdominal contents, in particular the liver and peritoneum. Patients who should be considered for diagnostic laparoscopy prior to laparotomy are those patients with possible undetectable metastatic disease, *i.e.*, primary tumors > 3 cm, marked weight loss, equivocal radiological findings, and elevated levels of carbohydrate antigen 19-9 (CA19-9)[10].

Definition and ambiguity of LAPC

The biology of LAPC is unique in that the tumor is confined locoregionally, without evidence of distant macrometastatic disease. The precise molecular mechanisms responsible for this behavior are unclear, but involve a preservation of the epithelial cell type *vs* de-differentiating into the mesenchymal phenotype responsible for distant spread[11]. Specific signals involved in this cell-type transformation include transforming growth factor beta (TGFβ), E-cadherin, N-cadherin, K-ras, and Snail, along with the chemokine CXCL12[12-14]. On a macroscopic level, LAPC has an anatomic definition and is represented by two subclasses of aggressive pancreas cancer - borderline resectable LAPC and unresectable LAPC. For the patient with LAPC, there is some level of involvement of the surrounding vascular structures, which include the superior mesenteric artery (SMA), celiac axis, hepatic artery, superior mesenteric vein (SMV), or portal vein (PV). Depending on the extent of vessel involvement, and whether the associated vascular structures are amenable to reconstruction in conjunction with resection of the tumor, defines whether the LAPC is deemed borderline resectable or unresectable (Figures 1 and 2).

Unfortunately, this definition of resectability has historically been vague, as there is considerable debate and controversy as to which patients are truly deemed resectable. Factors that contribute to this confusion are multiple, and include subjective interpretation of cross-sectional imaging, technical/surgical ability, and overall institutional experience. Because of the lack of consensus of a true definition of LAPC, the literature available for LAPC is not standardized, and generalizations and conclusions about the management of LAPC have suffered[5,8].

To address the lack of general consensus on a definition of LAPC, three guideline statements have recently been proposed. These include guideline proposals by the National Comprehensive Cancer Network (NCCN), The University of Texas M.D. Anderson Cancer Center (MDACC), and Americas Hepato-Pancreato-Biliary Association (AHPBA). All three guidelines include the aforementioned tumor relationships to vascular structures, however there is variability in the definition of the tumor-vascular involvement. Further, some guidelines have added additional subset criteria to more specifically define the population of patients with LAPC. The MDACC guidelines were supplemented with three sub-

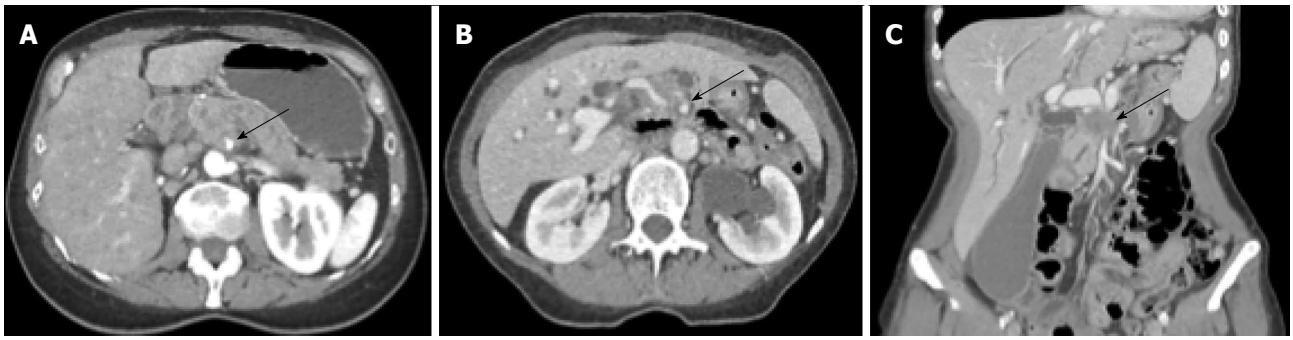

Figure 1 Computed tomography of locally advanced pancreatic cancer. Encasement is defined as greater than 180-degree involvement of the major vessels. A: Celiac axis is encased by locally advanced pancreatic cancer (arrow); B: Superior mesenteric artery and the replaced right hepatic artery are encased by pancreatic cancer (arrow); C: The portal vein and its confluence with splenic vein are encased by pancreatic cancer (arrow).

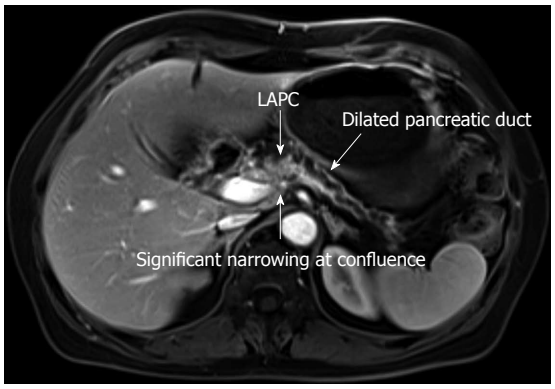

Figure 2 Magnetic resonance imaging of locally advanced pancreas cancer with vascular invasion and dilated pancreatic duct. LAPC: Locally advanced pancreatic cancer.

classifications of borderline resectable-types A, B and C. MDACC type A patients are only those patients with local, tumor-artery abutment. Type B patients are those with questionable extrapancreatic metastatic disease. Further defined, these type B patients are considered "oncologically borderline resectable" secondary to prior exploration which the original tumor was considered unresectable, a prior biopsy confirmed regional lymph node metastasis, or there is imaging concerning for liver metastases or high CA19-9. Type C patients are those defined as having a marginal pretreatment performance status[15].

The Alliance for Clinical Trials in Oncology (Alliance) recently initiated a multi-institutional trial to examine the use of neoadjuvant for LAPC in a single arm pilot study[16]. This study also seeks to address the lack of standardization in the definition of LAPC and to establish a research infrastructure that will create consensus around what constitutes borderline and unresectable LAPC. In the Alliance proposal, the definition of a borderline resectable pancreas cancer has an objective description of the tumor-vascular relationships, while omitting more subjective terms like abutment and encasement. These guidelines should create uniformity in how investigators define LAPC both for protocol and non-protocol based therapies[16] (Table 1).

A multi-disciplinary approach is highly recommended in the treatment of patients with LAPC, and can assist with arriving at a consensus recommendation for the treatment of patients with advanced disease. By bringing together medical oncologists, surgeons, radiologists, radiation oncologists, and other patient advocates, treatment plans for the patient with LAPC can be discussed and planned[17]. The complexity of LAPC is best managed by this multidisciplinary team of physicians working in concert to deliver individualized care for each patient[18]. The importance of a multi-modal, inter-disciplinary approach has been demonstrated in our own multidisciplinary pancreatic cancer clinic at Johns Hopkins, where we noted that 25% of patients seen in this setting had a significant change in their diagnosis or treatment[18].

BORDERLINE LAPC

Surgical resection of LAPC

Resection of the surrounding vascular structures for LAPC has been described since the 1970s. Fortner *et al*[19] described these "regional pancreatectomies" as type 1 (venous resection) and type 2 (arterial resection). These early reports demonstrated significant morbidity and mortality, and given the potential for likely systemic disease, combined tumor and vascular resection fell out of favor[20]. Despite early hesitation with combined resection of tumor and surrounding vascular structures, there is now growing enthusiasm for these more aggressive surgeries. One of the most controversial topics for these patients is the role of margin status after resection. This is particularly relevant for the patient with borderline LAPC, as vascular involvement of surrounding structures, even when technically achievable, may predispose to a positive resection margin.

Multiple reports suggest that margin status after resection of pancreas cancer influences survival[21,22]. However, other data demonstrate that margin status does not correlate with survival[23,24]. There are a variety of factors that have led to this ambiguity. One of the strongest influences fueling this discrepancy has been the lack of standardization of pathologic technique, *i.e.*, truly defining a "positive microscopic margin."[25]. This is evident from multiple large studies which demonstrate the rate of R1 involvement for pancreas cancer varies between

Table 1 Difference of definitions of anatomic borderline resectable pancreatic cancers from different sources

Tumor-vessel relationship on computed tomography	NCCN	MDACC	AHPBA/SSO/SSAT	Alliance
Superior mesenteric vein/ portal vein	Severely narrowed or occluded with possibility of reconstruction	Occluded with possibility of reconstruction	Abutment or encasement or occlusion with possibility of reconstruction	Interface between tumor and vessel > 180°, and or reconstructable
SMA	Abutment	Abutment	Abutment	Interface between tumor and vessel < 180°
Celiac axis	No abutment or encasement	Abutment	No abutment or encasement	Reconstructable interface
Common hepatic artery	Abutment or short segment encasement	Abutment or short segment encasement	Abutment or short segment encasement	Interface between tumor and vessel < 180°

Abutment, ≤ 180° or ≤ 50% of the vessel circumference; encasement, ≥ 180° or ≥ 50% of the vessel circumference. MDACC: Anderson Cancer Center; NCCN: National Comprehensive Cancer Network; AHPBA: Hepato-Pancreato-Biliary Association; SMA: Superior mesenteric artery.

20% and 80%, despite other clinicopathological variables being similar[26,27]. Fortunately, there have been improvements in standardization, and consensus is growing in the pathology community regarding how to examine the pathology specimen[28].

Other groups have also examined the effect of margin status from the surgical perspectives. Butturini *et al*[29] pooled hazard ratios of the effects of adjuvant therapy for resected patients, and compared the disease specific survival with their margin status. As part of their subset analysis, the authors concluded that resection margin (R0 *vs* R1) involvement was not a statistically significant prognostic factor, with a median survival of 14.1 mo for patients with an R1 resection compared with 15.9 mo for patients with R0 resections ($P = 0.24$).

From a technical standpoint, superior mesenteric vein and portal vein involvement by LAPC can be performed safely if resected and reconstructed at high-volume centers[30]. Reconstruction of the SMV/PV can be performed in a variety of ways depending on the degree of involvement. Patch or primary closure can be done for partial involvement, with patch reconstruction often done using the greater saphenous vein. Segmental reconstruction of the SMV can be performed with an interposition vein graft using the internal jugular, renal vein or superficial femoral vein[31,32]. Raut *et al*[24] examined 360 patients after pancreatectomy, of which 130 underwent SMV/PV reconstruction. Those patients who underwent vascular reconstruction had more R1 than R0 resections compared with those that did not have vascular reconstruction (HR = 2.00, $P = 0.015$). However, on multivariate analysis, there was no difference in survival between the R1 and R0 groups, leading the authors to conclude that not only was there no difference in patient survival based on R status, but venous reconstruction also did not predispose to worse disease-specific survival.

Compared with venous reconstruction, arterial involvement is probably more technically demanding. If an interposition graft is required, this can be done with polytetrafluoroethylene (PTFE) graft or saphenous vein[33]. Bockhorn *et al*[34] has reported one of the largest series to examine pancreatic resection with simultaneous arterial resection and reconstruction ($n = 29$); these authors found no difference in overall disease specific survival for patients who underwent arterial reconstruction *vs* those patients that had pancreatectomy alone (14.0 mo *vs* 15.8 mo respectively, $P = 0.152$). Both resection groups independently had better survival than the non-resected patients who only underwent palliative bypass (7.5 mo, $P < 0.05$ for both groups)[34].

Therefore, if feasible, most surgeons would recommend possible surgical resection for patients with borderline LAPC, with the goal of an R0 resection for all cases. While vascular resection with reconstruction is safe, patient selection is paramount. Those patients who cannot tolerate combined pancreatectomy and vascular reconstruction would benefit more from palliative bypass or no surgery at all.

BORDERLINE LAPC AND NEOADJUVANT THERAPY

Because of the dismal prognosis of pancreatic cancer, in particular those with borderline LAPC which may have a more aggressive biology, there is a growing body of literature to suggest that there is a potential role for neoadjuvant therapy to treat micrometastatic disease with chemotherapy, as well as treat local disease with radiation[35,36]. The rationale for neoadjuvant therapy for patients with borderline and LAPC is multifold. First, the chance of delivering full-dose chemotherapy with or without radiation is much better if given prior to surgery because of the potential delay in getting to treatment after a complex pancreatic resection. Second, neoadjuvant therapies provide insight into the biology of the disease, and can spare patients who progress or develop distant metastasis during treatment from undergoing a major surgery that would not be curative. Next, neoadjuvant therapies have the potential to downstage borderline resectable disease to the point of not requiring vascular reconstruction and/or increasing R0 resection. Lastly, preoperative therapy could be more effective than post resection therapy because the resected tumor bed may have decreased oxygenation and decreased drug delivery[37]. While there are benefits of neoadjuvant therapy for borderline LAPC,

these benefits must be weighed against the risks, which include delaying time to potentially curative surgery and significant time and side-effects for patients with limited life expectancies.

There are only retrospective studies with subsets of borderline LAPC, and a few smaller prospective studies examining the role of neoadjuvant therapies for borderline LAPC[15,38-40]. Patel *et al*[41] prospectively examined 17 patients with borderline LAPC for patients that were treated with combined chemoradiation, with 64% proceeding to surgery with 89% achieving an R0 resection. Stokes *et al*[40] also prospectively examined 40 borderline LAPC, also with combined chemoradiation, with 40% of patients proceeding to surgery, with 88% with an R0 resection, and median survival at 23 mo.

INITIALLY UNRESECTABLE LAPC AND NEOADJUVANT THERAPY

For initially unresectable LAPC, *i.e.*, those tumors with significant vascular involvement that involves a significant portion of the SMV or SMA, neoadjuvant therapy should be offered, and the tumor should be assessed for possible response and eventual resection. The efficacy of neoadjuvant therapy with this approach as a bridge to potential curative resection is broad, ranging from 3%-79%[42-44]. The different modalities of neoadjuvant therapy include single or multi-agent chemotherapy combined with radiation, chemotherapy alone, and chemotherapy followed by chemotherapy with radiation.

Combined chemotherapy with radiation

5-flourouracil (5-FU) infusion with radiation therapy has shown utility in many gastrointestinal cancers, and is used in the management of unresectable LAPC. One of the first studies to demonstrate the synergistic effects of 5-FU with radiation was the Gastrointestinal Study Group (GITSG) trial in 1981 that prospectively examined unresectable LAPC patients, randomly assigning 106 patients to three different treatments: radiation (60 Gy) alone, *vs* concurrent radiation (40 Gy) plus bolus 5-FU, *vs* higher dose concurrent radiation (60 Gy) plus bolus 5-FU[45]. The radiation alone group demonstrated poor 1-year survival (11%) *vs* 36% in the higher dose concurrent radiation group, and 38% in the concurrent lower radiation group. Other trials have demonstrated this synergistic and radiosensitizing effect of combined 5-FU with radiation[46-48]. Contrary to successes of these groups and the GITSG trials using combined 5-FU with radiation, a trial from the Eastern Cooperative Oncology Group (ECOG) randomized 91 patients with unresectable LAPC to either radiation (40 Gy) plus concurrent bolus 5-FU, followed by weekly maintenance 5-FU, *vs* 5-FU alone, and found no differences in survival (8.2 mo *vs* 8.3 mo)[49,50]. Despite the conflicting success of combined 5-FU/radiation therapy, this radiosensitization treatment modality has become an established approach to management of the patient with LAPC[51].

In an effort to capitalize on the benefits of combined 5-FU and radiation therapies, yet avoid the toxic side effects of 5-FU therapy, the oral formulation of 5-FU, capecitabine, has been introduced into many trials. To date there are multiple studies, albeit only a few prospective trials, that demonstrate that capecitabine can effectively replace infusional 5-FU in the setting of LAPC[52-54].

As the potential utility of combined 5-FU/radiation therapies was being recognized for LAPC, gemcitabine based regimens were gaining acceptance in the management of metastatic pancreas cancer[55]. Therefore, gemcitabine combined with radiation gained interest as a potential agent to study in the management of LAPC. Unfortunately, early phase I trials using gemcitabine with radiation were fraught with toxicities unlike the 5-FU based therapies, and required improvements in delivery of radiation[56-58]. As the toxicities of combined gemcitabine and radiation therapy became more manageable, studies were designed to compare the established 5-FU and radiation therapy with gemcitabine combined with radiation for LAPC.

Three large prospective studies were designed with this hypothesis in mind. The Federation Francophone de Cancerologie Digestive and Societie Francaise de Radiotherapie Oncologique (FFCD-SFRO) trial published in 2008 showed improved survival for those patients treated with gemcitabine alone *vs* combined radiotherapy with 5-FU (13.0 mo *vs* 8.6 mo, $P = 0.03$)[59]. The ECOG E4201 study, published 3 years after the FFCD-SFRO study, compared gemcitabine plus radiation with gemcitabine alone, and found improved survival in the combined group (11.1 mo *vs* 9.2 mo, $P = 0.017$), although there was more toxic side effects in the combined group[60]. The Taipei trial, which compared combined gemcitabine and radiation with combined 5-FU and radiation, concluded that combined gemcitabine and radiation therapy had improved overall survival (14.5 mo *vs* 6.7 mo, $P = 0.027$)[48]. These large series solidified the utility of gemcitabine based chemoradiation as an acceptable option for patients with LAPC.

A recent trial has further examined 5-FU combined therapies using capecitabine, and compared efficacy with gemcitabine-based chemoradiotherapy. Mukherjee *et al*[61] in the Selective Chemoradiation in Advanced Localized Pancreatic Cancer (SCALOP) study, examined 74 patients with LAPC who were randomly assigned gemcitabine or capecitabine. These authors found that the capecitabine treated patients had improved survival over the gemcitabine treated patients (15.2 mo *vs* 13.4 mo, $P = 0.012$). Furthermore, the gemcitabine treated patients had more toxic non-hematologic (10 *vs* 4, $P = 0.12$) and hematologic side effects (7 *vs* 0, $P = 0.008$).

Just as the combined chemotherapy and radiation algorithm has focused on changing the chemotherapeutic agent in an attempt to maximize survival benefit and minimize toxicity, other studies have examined the different radiation delivery modalities. The earlier combined chemoradiation treatments incorporated external beam

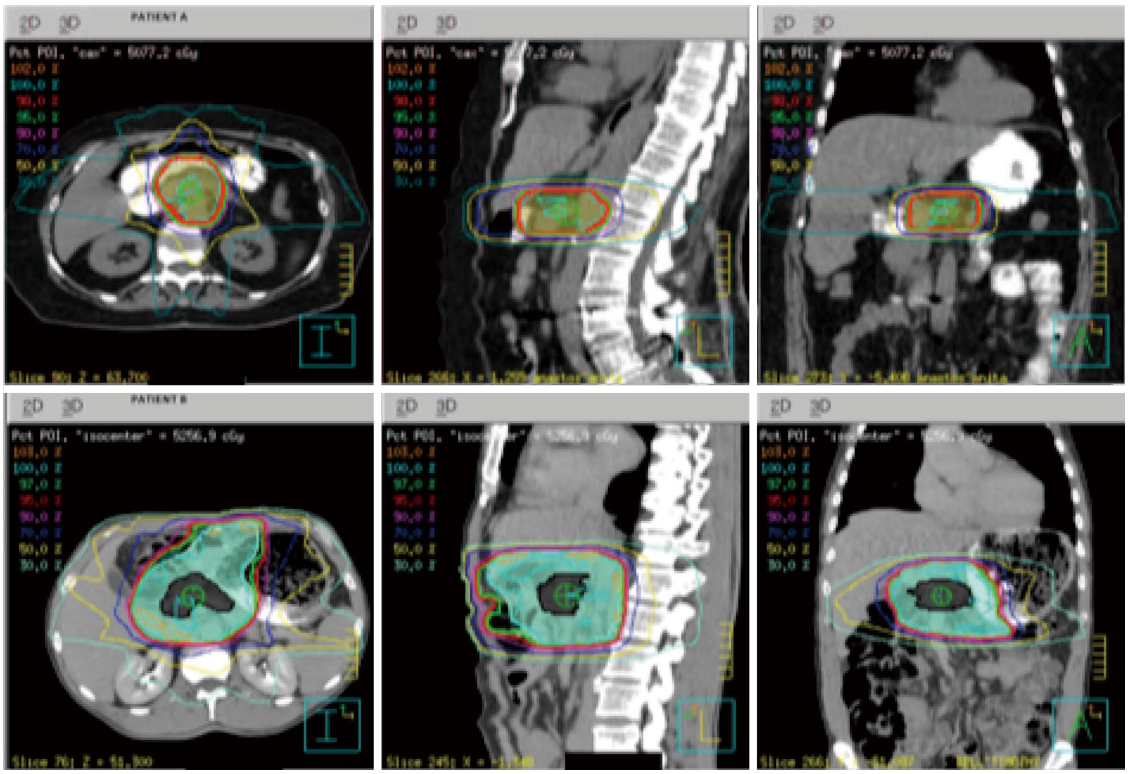

Figure 3 Depiction of stereotactic body radiation plan using computed tomography. Typically the tumor is expanded 2-3 mm to account for set up error microscopic extension and set-up error planning treatment volume. In the lower panel, (patient b) this represents a plan integrating intensity modulated radiation therapy (IMRT) where the tumor is expanded 1-3 cm to cover the tumor and peripancreatic lymph nodes. Stereotactic body radiation is often delivered over 1-5 d without chemotherapy. IMRT is delivered over 5-6 wk with concurrent chemotherapy.

radiation (EBRT). Since the 1980s, other delivery systems have developed with the integration of 3-D conformal radiation and subsequently intensity modulated radiation therapy (IMRT) and stereotactic body radiation (SBRT). Conventional EBRT has limitations in the amount of radiation that can be delivered to the pancreas tumor secondary to damage to the surrounding GI tract and other healthy tissues. In addition, EBRT also usually requires a large number of treatments given over 5-6 wk. SBRT and IMRT can deliver more focused radiation therapy to the tumor plus a margin, and thus limit dose to normal bowel resulting in less toxicity and dose escalation to the tumor. IMRT represents a further advancement from conformal EBRT. By utilizing 3-D conformations of a tumor target, radiation *via* IMRT can be delivered in smaller divisions of beams (beamlets), while both sparing healthy tissue and having the capacity to up or down regulate the intensity of the target directed beamlets[62]. SBRT enables delivery of even more precise and large doses of radiation to the pancreas tumor plus a small margin (usually 2-3 mm) because of the rapid dose fall-off beyond the treated volumes. SBRT is also usually given in 1-5 fractions, far fewer than EBRT (10-30)[63] (Figure 3).

Because of the toxicities which may arise during chemoradiation, combined with the overall poor survival of LAPC, it is critical in the multidisciplinary management of LAPC to identify which patients may experience worse outcomes. Rudra *et al*[64] identified pretreatment performance status and CA19-9 levels, along with treatment interruption as prognostic factors for patients with LAPC treated with chemoradiation. These authors proposed that patients should be identified with these poor outcome features prior to treatment, and consider other therapies such as chemotherapy alone or supportive care for patients with poor performance status.

Chemotherapy alone

Chemotherapy alone represents another management strategy for unresectable LAPC. The primary chemotherapy only regimens include gemcitabine alone; gemcitabine doublet therapy with oxaliplatin, cisplatin, erltoinib, or capecitabine; or triplet therapy with oxaliplatin and erlotinib, or oxalplatin and bevacizumab. Other non-gemcitabine-based regimens include irinotecan with docetaxel[65].

Multiple trials have examined patients with LAPC, comparing gemcitabine alone with various gemcitabine doublet therapies. Louvet *et al*[66], in the GERCORD and GISCAD trials found no difference in overall survival (9.0 mo *vs* 7.1 mo, $P = 0.13$) using gemcitabine alone *vs* doublet therapies. Similar survival was also seen when gemcitabine was compared with and without tipifanib (193 d *vs* 182 d, $P = 0.75$)[67]. Other groups have examined gemcitabine combined with irinotecan (IRINOGEM), and while time-to-progression initially showed promise for the IRINOGEM treated group *vs* gemcitabine alone

Table 2 Summary of recent chemotherapy trials for locally advanced pancreatic cancer

CHEMO trials	Component	Median survival	*P* value
GERCORD/GISCAD[66]	Gem ± oxaliplatin	9.0 mo *vs* 7.1 mo	0.13
Van Cutsem *et al*[67]	Gem ± tipifarnib	193 d *vs* 182 d	0.75
IRINOGEM[68]	Gem ± irinotecan	6.3 mo *vs* 6.6 mo	0.79
Von Hoff *et al*[69]	Gem ± *nab*-paclitaxel	8.5 mo *vs* 6.7 mo	< 0.001
PRODIGE[70]	Gem *vs* FOLFIRINOX	6.8 mo *vs* 11.1 mo	< 0.001

CHEMO: Chemotherapy; Gem: Gemcitabine.

(median 7.7 mo *vs* 3.9 mo, *P* value not reported), there was no difference in overall survival (6.3 mo *vs* 6.6 mo, $P = 0.789$)[68]. Von Hoff *et al*[69] using combined gemcitabine with nab-paclitaxel *vs* gemcitabine monotherapy demonstrated a survival benefit in patients with metastatic pancreas cancer (8.5 mo *vs* 6.7 mo, $P < 0.001$). The application of this regimen for LAPC is not known. In summary for gemcitabine-based chemotherapies, in the setting of LAPC, there are no prospective data to suggest that gemcitabine doublet, or even triplet therapy improves overall survival over monochemotherapy using gemcitabine alone.

While multiple agent gemcitabine based chemotherapies have not shown direct promise in the management of LAPC, other non-gemcitabine based regimens are being explored. The multiple agent therapy of 5-FU/leucovorin, irinotecan, and oxaliplatin (FOLFIRINOX) has recently shown promise in the management of metastatic pancreas cancer in the PRODIGE trial, and is being studied in the context of LAPC[70]. In three retrospective reviews of FOLFIRNOX for LAPC, partial response rates ranged from 25%-40%[71-73]. Other multiple agent therapies like oxaliplatin, 5-FU, and folinic acid (FOLFOX-6), and agents like 5-FU plus leucovorin plus irinotecan (FOLFIRI), are also being studied as potential agents to improve outcomes in unresectable LAPC[74,75]. While some progress has been shown using chemotherapy alone regimens for LAPC, the specific treatment with best results has yet to be determined (Table 2).

Chemotherapy followed by chemoradiotherapy

An additional treatment algorithm for LAPC is the use of chemotherapy followed by chemoradiotherapy. The specific goal of this treatment is to select the patients treated with chemotherapy who will benefit from chemoradiotherapy, and also to select those who have not progressed following the initiation of chemotherapy. The earliest and one of the largest studies to examine this mode of therapy was the Groupe Cooperatuer Multidsisciplinaire en Oncologie (GERCOR). This group retrospectively reviewed 181 patients with LAPC who had been treated with gemcitabine-based chemotherapy followed by chemoradiotherapy using 5-FU in continuous infusion[76]. Fifty-three patients developed metastases in the first 3 mo of chemotherapy and were subsequently not eligible for chemoradiation. In the remaining 128 patients who did not progress, 56 continued with chemotherapy alone with overall survival of 11.7 mo. The other 72 patients received chemoradiation, with overall survival of 15.0 mo ($P < 0.01$).

Another retrospective study by the University of Texas M.D. Anderson Cancer Center examined consecutive patients with LAPC who had received treatment with chemoradiation or induction chemotherapy followed by chemoradiotherapy[77]. Of the 323 patients in this study, 76 received a median of 2.5 mo of gemcitabine prior to chemoradiation. Those who underwent chemotherapy prior to combined chemoradiation had improved median overall survival (11.9 mo *vs* 8.5 mo, $P < 0.001$), and also demonstrated improved progression free survival (6.4 mo *vs* 4.2 mo, $P < 0.001$).

While the use of chemotherapy followed by chemoradiation has shown early promise in the management of LAPC, phase Ⅱ/Ⅲ studies are needed. The ECOG 1200 phase Ⅱ trial was initially designed to evaluate the safety of borderline resectable LAPC using the algorithm of chemotherapy followed by chemoradiation, but was closed early because of low recruitment[44].

In summary of the treatments modalities available for unresectable LAPC, a recent retrospective review by Lloyd *et al*[65] compared outcomes based on combined chemotherapy with radiation, chemotherapy alone, and chemotherapy followed by chemotherapy with radiation. While the sample size was small ($n = 115$), and included borderline and unresectable LAPC, the authors concluded on multivariate analysis that chemotherapy followed by chemotherapy with radiation was associated with improved overall survival over chemotherapy alone or combined chemotherapy with radiation (median survival 21.5 mo *vs* 13.9 mo and 12.5 mo respectively, $P < 0.05$).

Locoregional therapy with irreversible electroporation

For some patients with LAPC, irreversible electroporation (IRE) has shown promise in downstaging and prolonging survival. IRE is a non-thermal modality that uses high voltage and low energy direct current to increase cell membrane permeability and effectively create defects in cell membranes, resulting in loss of homeostasis and subsequent cell death. IRE has minimal effect on blood vessel scaffolding, which is crucial and particularly relevant for LAPC, as surrounding vascular involvement may be present[78,79].

The NanoKnife®IRE system has been commercially available since 2009 and is FDA-approved to treat soft tissue tumors. The safety of IRE use in the pancreas has been shown in swine models with rapid resolution

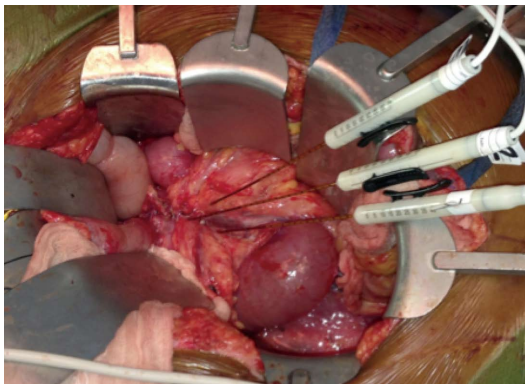

Figure 4 An intraoperative image of *in situ* irreversible electroporation being used in a patient with locally advanced pancreatic cancer. Three probes are placed around the tumor which is encasing the superior mesenteric vein causing complete occlusion plus superior mesenteric artery involvement.

of pancreatitis and preservation of vascular structures. Ablation effects can be achieved at a median size of 3 cm with 3000 volts setting of the NanoKnife®IRE system[78]. Usually, 2-4 probes of the NanoKnife®IRE system are used to treat LAPC. The probes are placed using intra-operative ultrasound guidance. In a retrospective series of patients treated at a single institution, Martin *et al*[80]. applied this new device and demonstrated in unresectable LAPC that IRE can improve both local (14 mo *vs* 6 mo, $P = 0.001$) and distant progression free survival (15 mo *vs* 9 mo, $P = 0.02$), compared with systemic therapy and chemoradiation. Overall survival for patients treated with IRE was also improved compared with patients treated with chemotherapy alone or chemoradiation (20 mo *vs* 13 mo, $P = 0.03$, exact chemoradiation regimens not specified) (Figures 4 and 5).

IRE can be administered percutaneously under imaging guidance, thereby avoiding the morbidity of a laparotomy. Narayanan *et al*[81] reported the results of 11 patients treated with IRE for LAPC. In this study, prior to IRE, all patients had received some form of chemoradiation, though the exact regimen was not specified. Patients were selected for IRE if they were not candidates for, or were intolerant of chemotherapy or radiation. The procedure was performed under general anesthesia, with CT guidance, and electrodes were placed at a maximum of 2.2 cm apart. Post treatment, all patients demonstrated patent vasculature in the treatment zone and there were no deaths related to the procedure. Two patients underwent partial responses leading to eventual resection 4 and 5 mo post IRE, with one of these patients demonstrating a complete response. Both patients remained disease free at 11 and 14 mo. At our institution, we often maximize both systemic and local therapy (radiation), then in well selected patients, we attempt surgical resection with IRE in an attempt to sterilize surgical margins or treat the tumor intra-operatively if found to be unresectable.

CONCLUSION

LAPC is a biologically aggressive cancer with unique characteristics, prognosis, and management strategies that differentiate this pancreatic tumor from resectable cancer and metastatic disease. The only means to potentially cure LAPC is by maximizing upfront systemic and local therapy followed by a margin negative surgical resection. At Johns Hopkins Hospital, we recommend tailoring therapy to maximize the chance to offer the patient a chance at surgical resection. In general, if LAPC is pre-operatively identified as not resectable, then we proceed down a pathway of local control with radiation therapy combined with systemic control with chemotherapy. After chemoradiation, we restage and re-evaluate for possible resection, with IRE as an alternative therapy for the unresectable LAPC.

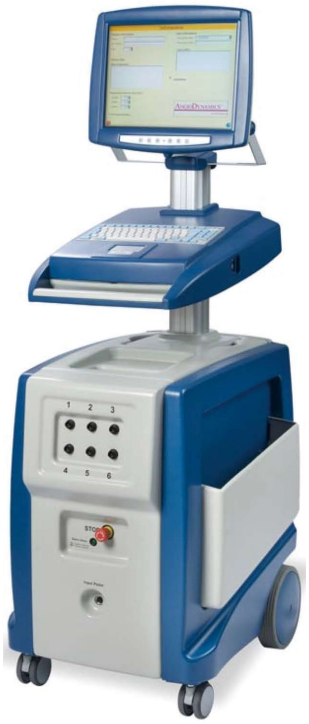

Figure 5 This is the representative base unit and generator for irreversible electroporation, manufactured by AngioDynamics, Latham, NY.

Unfortunately, surgical and chemoradiation protocols have suffered from lack of consensus on what truly defines both a resectable LAPC and a positive resection margin. But with growing adoption of consensus guidelines, and the incorporation of improved systemic therapies and local therapeutic options with decreased side effects, progress is being made in identifying which patients with LAPC can truly benefit from surgical resection.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Systematic review of novel ablative methods in locally advanced pancreatic cancer

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Abstract

Unresectable locally advanced pancreatic cancer with or without metastatic disease is associated with a very poor prognosis. Current standard therapy is limited to chemotherapy or chemoradiotherapy. Few regimens have been shown to have a substantial survival advantage and novel treatment strategies are urgently needed. Thermal and laser based ablative techniques are widely used in many solid organ malignancies. Initial studies in the pancreas were associated with significant morbidity and mortality, which limited widespread adoption. Modifications to the various applications, in particular combining the techniques with high quality imaging such as computed tomography and intraoperative or endoscopic ultrasound has enabled real time treatment monitoring and significant improvements in safety. We conducted a systematic review of the literature up to October 2013. Initial studies suggest that ablative therapies may confer an additional survival benefit over best supportive care but randomised studies are required to validate these findings.

Key words: Pancreatic cancer; Radiofrequency ablation; Photodynamic therapy; Cryoablation; Microwave ablation; High frequency focused ultrasound; Irreversible electroporation

Core tip: Unresectable locally advanced pancreatic cancer with or without metastatic disease is associated with a very poor prognosis. Current standard therapy is limited to chemotherapy or chemoradiotherapy. Few regimens have been shown to have a substantial survival advantage and novel treatment strategies are urgently needed. Initial studies of ablation in the pancreas were associated with significant morbidity and mortality, which limited widespread adoption. Modifications to the various applications, in particular combining the techniques with high quality imaging such as computed tomography and intraoperative or endoscopic ultrasound has enabled real time treatment monitoring and significant improvements in safety.

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BACKGROUND

Pancreatic ductal adenocarcinoma (PDAC) is the tenth most common cancer in the UK but the fifth commonest

cause of cancer death. At diagnosis more than 80% of patients have locally advanced or metastatic disease and are unsuitable for curative surgical resection. Prognosis in pancreatic cancer is dismal; median survival for locally advanced disease is just 6-10 mo, however in patients with metastatic disease this falls to 3-6 mo. Overall 5 year survival is less than 4%[1].

Standard options available for treating patients with inoperable PDAC are limited to chemotherapy, radiotherapy, or a combination of the two. Gemcitabine is the most commonly used chemotherapy agent in pancreatic cancer, however recent studies have shown that in combination with other chemotherapy agent's further improvements in overall survival can be gained. A recent randomised Phase Ⅲ study (GemCap) reported a median survival in the combination gemcitabine + capecitabine group of 7.1 mo compared with 6.2 mo in those who received gemcitabine alone. The 1-year overall survival (OS) rates were 24.3% for combination therapy and 22% for gemcitabine alone (HR = 0.86, 95%CI: 0.72-1.02, $P = 0.077$)[2]. A further large European study compared gemcitabine to FOLFIRINOX (fluorouracil, leucovorin, irinotecan and oxaliplatin) and demonstrated a significant survival advantage in the FOLFIRINOX group compared with gemcitabine alone (median 11.1 mo *vs* 6.8 mo)[3]. The phase Ⅲ MPACT study found that weekly intravenous nab-paclitaxel with gemcitabine resulted in a significantly higher overall survival compared to gemcitabine monotherapy (8.5 mo *vs* 6.7 mo, HR = 0.72, $P < 0.0001$)[4].

Given that so few patients with PDAC are suitable for curative surgery and most have only a limited response to chemotherapy; tumour debulking or interstitial ablation has been investigated as a potential additional therapy. A recent systematic review compared R2 resections to palliative bypass alone in the management of advanced PDAC. A small non-significant survival advantage was observed in the R2 resection group; 8.2 mo compared to 6.7 mo in the palliative bypass group. However patients undergoing R2 resections had a significantly higher morbidity (RR = 1.75, 95%CI: 1.35-2.26, $P < 0.0001$), mortality (RR = 2.98, 95%CI: 1.31-6.75, $P = 0.009$) and longer hospital stay (mean difference, 5 d, 95%CI: 1-9 d, $P = 0.02$), hence R2 resections are not recommended as part of the standard management of PDAC[5]. However minimally invasive ablative therapies delivered percutaneously or endoscopically have become part of standard therapy in many other solid organ tumours, particularly in patients with inoperable disease or who are unfit for surgical resection[6]. Early studies of local ablation in the pancreas were associated with high morbidity and mortality[7]. However improvements in delivery and in particular combining the technology with high quality real-time imaging, has reduced associated complications. The safety and efficacy of each ablative therapy in non-operable PDAC will be evaluated in this review.

RESEARCH METHODOLOGY

The primary aim of this review was to assess safety and efficacy of each ablation therapy in the treatment of locally advanced or metastatic PDAC. Secondary endpoints included improvements in overall survival, changes in symptoms, tumour markers or performance status where available. A systematic literature search was performed using the PubMed, EMBASE databases and the Cochrane Library for studies published in the English language up to 1st October 2013. MeSH terms were decided by a consensus of the authors and were (radiofrequency ablation, catheter ablation, photodynamic therapy, PDT, cryoablation, cryosurgery, laser, high intensity focused ultrasound ablation, microwave, electroporation) and (pancreas OR pancreatic), and were restricted to the title, abstract and keywords. Only articles, which described ablation in unresectable PDAC, were included. Articles that described the use of ablative therapies in premalignant pancreatic disease were excluded but outcomes are summarised in Table 1. Similarly studies that included non-ablative therapies were also excluded but have been summarised in Table 2. Any study with fewer than four patients and those reporting on tumours that did not originate in the pancreas were excluded. In cryoablation and high frequency focused ultrasound of the pancreas, many of the largest case-series are published in non-English language medical journals. Although articles not published in the English language were excluded from this systematic review, if an English language abstract was available the results were included in the summary tables. All references were screened for potentially relevant studies not identified in the initial literature search. The following variables were extracted for each report when available: number of patients, disease extent, device used and settings, distance of probe from surrounding structures, duration of therapy and number of ablations applied, additional safety methods used. Thirty-two papers were included (Figure 1).

THERMAL ABLATIVE TECHNIQUES

Radiofrequency ablation

Radiofrequency ablation (RFA) causes tissue destruction through the application of a high frequency alternating current that generates high local temperatures leading to a coagulative necrosis. The technique has been widely used in many solid organ malignancies and is now part of standard therapy in several tumours including hepatocellular carcinoma[6]. The first application of RFA in the normal porcine pancreas was described in 1999. Although this application was performed under EUS guidance[8] it has nearly always been delivered intraoperatively (rarely percutaneously) in combination with palliative bypass surgery[9]. Although RFA was deemed to be feasible and safe in animal studies[8], early clinical applications in the pancreas were associated with unacceptably high rates of morbidity (0%-40%) and mortality (0%-25%) (Table 3)[7,10-14]. Most RFA of pancreatic tumours has been performed using the Cool-tip™ RF Ablation system (Radionics). Many of the complications arose as a result of inadvertent damage to structures adjacent to

Table 1 Use of ablative therapies to treat cystic and solid premalignant lesions of the pancreas

Author	Premalignant lesion	*n*	Treatment	Median area of ablation, mm (range)	Outcome	Complications
Gan *et al*[46]	Cystic tumours of the pancreas	25	EUS guided ethanol lavage	19.4 (6-30)	Complete resolution 35%	None
Oh *et al*[73]	Cystic tumours of the pancreas	14	EUS guided ethanol lavage + paclitaxel	25.5 (17-52)	Complete resolution in 79%	Acute pancreatitis (n = 1) Hyperamylasaemia (n = 6) Abdominal pain (n = 1)
Oh *et al*[74]	Cystic tumours of the pancreas	10	EUS guided ethanol lavage + paclitaxel	29.5 (20-68)	Complete resolution in 60%	Mild pancreatitis (n = 1)
DeWitt *et al*[75]	Cystic tumours of the pancreas	42	Randomised double blind study: Saline *vs* ethanol	22.4 (10-58)	Complete resolution in 33%	Abdominal pain at 7 d (n = 5) Pancreatitis (n = 1) Acystic bleeding (n = 1)
Oh *et al*[47]	Cystic tumours of the pancreas	52	EUS guided ethanol lavage + paclitaxel	31.8 (17-68)	Complete resolution in 62%	Fever (1/52) Mild abdominal discomfort (1/52) Mild pancreatitis (1/52) Splenic vein obliteration (1/52)
Levy *et al*[76]	PNET	8	EUS guided ethanol lavage (5 patients) and intra-operative ultrasound guided (IOUS) ethanol lavage (3 patients)	16.6 (8-21)	Hypoglycemia symptoms disappeared 5/8 and significantly improved 3/8	EUS guided: No complications. IOUS-guided ethanol injection: Minor peritumoral bleeding (1/3), pseudocyst (1/3), pancreatitis (1/3)
Pai *et al*[21]	Cystic tumours of the pancreas + neuroendocrine tumours	8	EUS guided RFA	Mean size pre RFA, 38.8 mm *vs* mean size post RFA, 20 mm	Complete ablation in 25% (2/8)	2/8 patients had mild abdominal pain that resolved in 3 d

RFA: Radiofrequency ablation; EUS: Endoscopic ultrasound; PNET: Pancreatic neuroendocrine tumour.

Table 2 Endoscopic ultrasound administered non-ablative anti-tumour therapies for pancreatic ductal adenocarcinoma

Author	Therapy	Patients	*n*	Outcome and survival	Complications
Chang *et al*[77]	Cytoimplant (mixed lymphocyte culture)	Unresectable PDAC	8	Median survival: 13.2 mo. 2 partial responders and 1 minor response	7/8 developed low-grade fever 3/8 required biliary stent placement
Hecht *et al*[78]	ONYX-015 (55-kDa gene-deleted adenovirus) + Ⅳ gemcitabine	Unresectable PDAC	21	No patient showed tumour regression at day 35. After commencement of gemcitabine, 2/15 had a partial response	Sepsis: 2/15 Duodenal perforation: 2/15
Hecht *et al*[79] Chang *et al*[80,81]	TNFerade (replication-deficient adenovector containing human tumour necrosis factor (TNF)-α gene)	Locally advanced PDAC	50	Response: One complete response, 3 partial responses. Seven patients eventually went to surgery, 6 had clear margins and 3 survived > 24 mo	Dose-limiting toxicities of pancreatitis and cholangitis were observed in 3/50
Herman *et al*[82]	Phase Ⅲ study of standard care plus TNFerade (SOC + TNFerade) *vs* standard care alone (SOC)	Locally advanced PDAC	304 (187 SOC + TNFerade)	Median survival: 10.0 mo for patients in both the SOC + TNFerade and SOC arms [hazard ratio (HR), 0.90, 95%CI: 0.66-1.22, P = 0.26]	No major complications. Patients in the SOC + TNFerade arm experienced more grade 1 to 2 fever than those in the SOC alone arm (P < 0.001)
Sun *et al*[83]	EUS-guided implantation of radioactive seeds (iodine-125)	Unresectable PDAC	15	Tumour response: "partial" in 27% and "minimal" in 20%. Pain relief: 30%	Local complications (pancreatitis and pseudocyst formation) 3/15. Grade Ⅲ hematologic toxicity in 3/15
Jin *et al*[84]	EUS-guided implantation of radioactive seeds (iodine-125)	Unresectable PDAC	22	Tumour response: "partial" in 3/22 (13.6%)	No complications

PDAC: Pancreatic ductal adenocarcinoma; EUS: Endoscopic ultrasound.

the zone of ablation such as the normal pancreas, duodenum, biliary tree or peri-pancreatic vasculature. These early studies applied high temperatures (> 90 ℃) and multiple rounds of ablation to treat large tumours in the head of the pancreas in one session[13]. An *ex-vivo* study of the thermal kinetic characteristics of RFA found that the optimal settings for RFA in the pancreas to prevent injury to the adjacent viscera was 90 ℃ applied for 5 min[15]. Subsequent clinical studies that reduced the RFA temperature from 105 ℃ to 90 ℃, reported only minimal RFA-related complications[7]. Active cooling of the major vessels and duodenum with saline during intraoperative RFA and observing at least a 0.5 cm area between the zone of ablation and major structures, reduced compli-

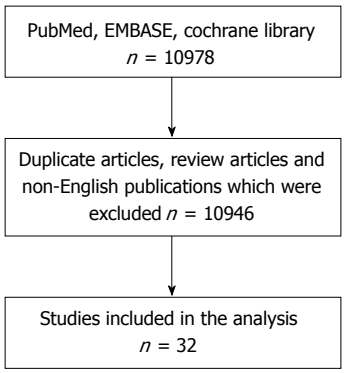

Figure 1 Systematic review schema.

cations[10,16,17]. Since most of the mortality resulted from uncontrollable gastrointestinal haemorrhage from ablated tumours in the head of the pancreas, some authors have recommended this probe should only be employed in body or tail tumours[10,16].

All studies have demonstrated that RFA leads to tumour necrosis and a decrease of tumour volume[9,12,17,18]. Some studies have also observed an improvement in tumour related symptoms, in particular a reduction of back pain and analgesia requirements. Tumour markers (carbohydrate antigen 19-9) also decrease following effective ablation[16]. Although all patients treated with RFA ultimately developed disease progression[9,11,12,17,18], when compared to patients with advanced disease who received standard therapy in a non-randomised cohort study, patients who received combination therapy had prolonged survival (33 mo *vs* 13 mo, $P = 0.0048$)[11]. However, this was a single centre study that only included 25 patients (12 receiving RFA). An earlier non-randomised study did not demonstrate the same survival advantage[12]. Spiliotis *et al*[11] also evaluated overall survival following RFA according to tumour stage. Patients with stage Ⅲ disease had a significant improvement in survival following RFA compared to patients with the same stage of disease receiving best supportive care ($P = 0.0032$). In contrast, no difference in overall survival was shown in patients with metastatic PDAC, following RFA treatment ($P = 0.1095$). Larger studies, in combination with systemic chemotherapy, would be needed to evaluate any potential role of RFA in patients with metastatic disease.

Recently two new RFA probes have been developed that can be placed down the working channel of an endoscope, enabling RFA to be administered under EUS guidance. Twenty-two patients with locally advanced PDAC were treated with the cryotherm probe (CTP) (ERBE Elektromedizin GmbH, Tübingen, Germany) that incorporates radiofrequency ablation with cryogenic cooling. The probe was sited successfully in 16 patients (72.8%); stiffness of the gastrointestinal wall and tumour prevented placement in the others. Following the procedure three patients reported mild abdominal pain and one experienced minor gastrointestinal bleeding, not requiring transfusion[19]. In a further study, 7 patients with unresectable PDAC received EUS guided RFA using the monopolar radiofrequency (RF) catheter (1.2 mm Habib EUS-RFA catheter, Emcision Ltd, London). The tumour was shown to decrease in size in all cases and only one patient developed mild pancreatitis[20]. Long-term follow up is not available on the efficacy of these new catheters. Early clinical studies have also used the Habib EUS RFA catheter to treat cystic tumours of the pancreas (Table 1)[21].

Microwave ablation

Microwave (MW) current is produced by a generator connected *via* a coaxial cable to 14-gauge straight MW antennas with a 3.7 cm or 2 cm radiating section. One or two antennae are then inserted into the tumour for 10 min. The largest case series of microwave ablation in locally advanced PDAC includes 15 patients. Although MW ablation can be performed percutaneously or intraoperatively[22], in this series it was performed intraoperatively at the time of palliative bypass surgery. All tumours were located in the head or body of the pancreas and had an average size of 6 cm (range 4-8 cm); none had distant metastasis on imaging. Partial necrosis was achieved in all patients and there was no major procedure-related morbidity or mortality. However minor complications were seen in 40% (mild pancreatitis, asymptomatic hyperamylasia, pancreatic ascites, and minor bleeding). The longest survival of an individual patient in this series was 22 mo[23].

Cryoablation

The successful use of cryoablation in the pancreas was first reported in primate experiments in the 1970s[24]. However its potential application as a therapy in pancreatic cancer was not described for a further 20 years[25]. Cryoablation is most commonly performed intraoperatively under ultrasound guidance. Small lesions (< 3 cm) can be reliably frozen with a single, centrally placed probe but larger tumours require the placement of multiple probes or sequential treatments. Most studies have used the argon-gas-based cryosurgical unit (Endocare, Inc., CA, United States) and employ a double "freeze/thaw" cycle. The tumour is cooled to -160 ℃ and the resulting iceball monitored with ultrasound to ensure the frozen region encompasses the entire mass and does not compromise local structures. The tissue is then allowed to slowly thaw to 0 ℃ and a second cycle of freezing is performed after any necessary repositioning of the cryoprobes. Like in many of the RFA studies, the authors advocated a 0.5 cm margin of safety from major structures and that ideally the procedure should be performed at the same time as palliative bypass surgery or endoscopic biliary and duodenal stenting. Ablation of liver metastases can also be performed simultaneously[26].

The largest experience of intraoperative and percutaneous cryoablation in pancreatic cancer has been reported from Asia. To date more than 200 patients with

Table 3 Studies of radiofrequency ablation in pancreatic ductal adenocarcinoma

Study	Patients	*n*	Route of administration	Device	RFA temp (℃)	RFA duration (min)	Outcome	Complications
Matsui *et al*[12]	Unresectable PDAC	20 LA:9 M:11	At laparotomy 4 RFA probes were inserted into the tumour 2 cm apart	A 13.56-MHz RFA pulse was produced by the heating apparatus	50	15	Survival: 3 mo	Mortality: 10% (septic shock and gastrointestinal bleeding)
Hadjicostas *et al*[14]	Locally advanced and unresectable PDAC	4	Intraoperative (followed by palliative bypass surgery)	Cool-tip™ RFAblation system	NR	2-8	All patients were alive one year post-RFA	No complications encountered
Wu *et al*[10]	Unresectable PDAC	16 LA:11 M:5	Intraoperative	Cool-tip™ RFAblation system	30-90	12 at 30 ℃ then 1 at 90 ℃	Pain relief: back pain improved (6/12)	Mortality: 25% (4/16) Pancreatic fistula: 18.8% (3/16)
Spiliotis *et al*[11]	Stage Ⅲ and Ⅳ PDAC receiving palliative therapy	12 LA:8 M:4	Intraoperative (followed by palliative bypass surgery)	Cool-tip™ RFAblation system	90	5-7	Mean survival: 33 mo	Morbidity: 16% (biliary leak) Mortality: 0%
Girelli *et al*[7]	Unresectable locally advanced PDAC	50	Intraoperative (followed by palliative bypass surgery)	Cool-tip™ RFAblation system	105 (25 pts) 90 (25 pts)	Not reported	Not reported	Morbidity 40% in the first 25 patients. Probe temperature decreased from 105°C to 90 ℃ Morbidity 8% in second cohort of 25 patients. 30-d mortality: 2%
Girelli *et al*[50]	Unresectable locally advanced PDAC	100	Intraoperative (followed by palliative bypass surgery)	Cool-tip™ RFAblation system	90	5-10	Median overall survival: 20 mo	Morbidity: 15%. Mortality: 3%
Giardino *et al*[51]	Unresectable PDAC. 47 RFA alone. 60 had RFA + radiochemotherapy (RCT) and/or intra-arterial systemic chemotherapy (IASC)	107	Intraoperative (followed by palliative bypass surgery)	Cool-tip™ RFAblation system	90	5-10	Median overall survival: 14.7 mo in RFA alone but 25.6 mo in those receiving RFA + RCT and/or IADC (P = 0.004)	Mortality: 1.8% (liver failure and duodenal perforation) Morbidity: 28%
Arcidiacono *et al*[19]	Locally advanced PDAC	22	EUS-guided	Cryotherm probe; bipolar RFA + cryogenic cooling	NR	2-15	Feasible in 16/22 (72.8%)	Pain (3/22)
Steel *et al*[41]	Unresectable malignant bile duct obstruction (16/22 due to PDAC)	22	RFA + SEMS placement at ERCP	Habib EndoHPB wire guided catheter	NR	Sequential 2 min treatments - median 2 (range 1-4)	Median survival: 6 mo Successful biliary decompression (21/22)	Minor bleeding (1/22) Asymptomatic biochemical pancreatitis (1/22), percutaneous gallbladder drainage (2/22). At 90-d, 2/22 had died, one with a patent SEMS
Figueroa-Barojas *et al*[42]	Unresectable malignant bile duct obstruction (7/20 due to PDAC)	20	RFA + SEMS placement at ERCP	Habib EndoHPB wire guided catheter	NR	Sequential 2 min treatments	SEMS occlusion at 90 d (3/22) Bile duct diameter increased by 3.5mm post RFA (P = 0.0001)	Abdominal pain (5/20), mild post-ERCP pancreatitis and cholecystitis (1/20)
Pai *et al*[20]	Locally advanced PDAC	7	EUS-guided	Habib EUS-RFA catheter	NR	Sequential 90s treatments - median 3 (range 2-4)	2/7 tumours decreased in size	Mild pancreatitis: (1/7)

PDAC: Pancreatic ductal adenocarcinoma; LA: Locally advanced PDAC; M: Metastatic PDAC; SEMS: Self-expanding metal stent; RFA: Radiofrequency ablation; EUS: Endoscopic ultrasound; ERCP: Endoscopic retrograde cholangiopancreatography.

Table 4 Studies of cryoablation in pancreatic ductal adenocarcinoma

Study	*n*	Patients	Study	Outcome	Complications
Patiutko *et al*[25] (non-English article)	30	Locally advanced PDAC	Combination of cryosurgery and radiation	Pain relief and improvement in performance status: 30/30	Not reported
Kovach *et al*[52]	9	Unresectable PDAC	Phase I study of intraoperative cryoablation under US guidance. Four had concurrent gastrojejunostomy	7/9 discharged with non-intravenous analgesia and 1/9 discharged with no analgesia	No complications reported
Li *et al*[53] (non-English article)	44	Unresectable PDAC	Intraoperative cryoablation under US guidance	Median overall survival: 14 mo	40.9% (18/44) had delayed gastric empting. 6.8% (3/44) had a bile and pancreatic leak
Wu *et al*[54] (non-English article)	15	Unresectable PDAC	Intraoperative cryoablation under US guidance	Median overall survival: 13.4 mo	1/15 patients developed a bile leak
Yi *et al*[55] (non-English article)	8	Unresectable PDAC	Intraoperative cryoablation under US guidance	Not reported	25% (2/8) developed delayed gastric emptying
Xu *et al*[26]	38	Locally advanced PDAC, 8 had liver metastases	Intraoperative or percutaneous cryoablation under US or CT guidance + (125) iodine seed implantation	Median overall survival: 12 mo. 19/38 (50.0%) survived more than 12 mo	Acute pancreatitis: 5/38 (one has severe pancreatitis)
Xu *et al*[56]	49	Locally advanced PDAC, 12 had liver metastases	Intraoperative or percutaneous cryoablation under US or CT guidance and (125) iodine seed implantation. Some patients also received regional celiac artery chemotherapy	Median survival: 16.2 mo. 26 patients (53.1%) survived more than 12 mo	Acute pancreatitis: 6/49 (one had severe pancreatitis)
Li *et al*[57]	68	Unresectable PDAC requiring palliative bypass	Retrospective case-series of intraoperative cryoablation under US guidance, followed by palliative bypass	Median overall survival: 30.4 mo (range 6-49 mo)	Postoperative morbidity: 42.9%. Delayed gastric emptying occurred in 35.7%
Xu *et al*[58]	59	Unresectable PDAC	Intraoperative or percutaneous cryotherapy	Median survival: 8.4 mo. Overall survival at 12 mo: 34.5%	Mild abdominal pain: 45/59 (76.3%) Major complications (bleeding, pancreatic leak): 3/59 (5%) 1/59 developed a tract metastasis
Niu *et al*[29]	36 (CT) 31 (CIT)	Metastatic PDAC	Intraoperative cryotherapy (CT) or cryoimmunotherapy (CIT) under US guidance	Median overall survival in CIT: 13 mo CT: 7 mo	Not reported

PDAC: Pancreatic ductal adenocarcinoma.

unresectable pancreatic cancer have undergone cryoablation alone or in combination with other therapies (Table 4). Effective control of pain, normalisation of CA 19-9, improvement in performance status and prolonged survival have all been reported following cryoablation. Rates of significant complications appear to be lower than in other methods of ablation. Although some patients did encounter delayed gastric emptying following the treatment, this commonly settled with conservative management within a few days. Studies to date are summarised in Table 5. The process has also been shown to initiate antiangiogenesis and a systemic immunological response, which may promote additional anti-tumour effects[27,28]. However evaluation through larger studies will be necessary to fully determine this effect.

Early clinical studies have also combined the administration of cryotherapy with immunotherapy. In a study of 106 patients with unresectable PDAC, 31 received cryoimmunotherapy, 36 cryotherapy, 17 immunotherapy and 22 chemotherapy. Median overall survival was higher in the cryoimmunotherapy (13 mo) and cryotherapy groups (7 mo) than in the chemotherapy group (3.5 mo; both $P < 0.001$) and was higher in the cryoimmunotherapy group than in the cryotherapy ($P < 0.05$) and immunotherapy groups (5 mo; $P < 0.001$)[29].

LASER BASED ABLATIVE THERAPY

Photodynamic therapy

Photodynamic therapy (PDT) results in tumour ablation by exposure to light following an intravenous injection of a photosensitiser [*e.g.*, *meso*-tetra(hydroxyphenyl)chlorin (mTHPC), porfimer sodium or verteporfin] which is taken up by cells. It leads to a predictable zone of ablation within the tumour. To date, light has been delivered *via* small optic fibers which have nearly always been positioned percutaneously under image guidance (*e.g.*, CT)[30-32]. However these fibers can pass through a 19G needle, so administration under endoscopic ultrasound guidance is feasible.

The first Phase I trial of PDT in locally advanced PDAC was conducted in 2002. Substantial tumour necro-

Table 5 Studies of photodynamic therapy in pancreatic ductal adenocarcinoma

Study	*n*	Study	Photosensitiser	Number of fibres	Number of ablations	Outcome and survival	Complications
Bown *et al*[30]	16	CT guided percutaneous PDT to locally advanced but inoperable PDAC without metastatic disease	mTH-PC	Single	1	Tumour necrosis: 16/16. Median survival: 9.5 mo. 44% (7/16) survived > 1 year	Significant gastrointestinal bleeding: 2/16 (controlled without surgery)
Huggett *et al*[31,32]	13 + 2	CT guided percutaneous PDT to locally advanced but inoperable PDAC without metastatic disease	Verteporfrin	Single (13) Multiple (2)	1	Technically feasible: 15/15. Dose dependent necrosis occurred	Single fibre: No complications. Multiple fibres: CT evidence of inflammatory change anterior to the pancreas, no clinical sequelae

PDAC: Pancreatic ductal adenocarcinoma; CT: Computed tomography.

sis was achieved in all 16 patients included in the study. Median survival after PDT was 9.5 mo (range 4-30 mo). 44% (7/16) were alive one year after PDT. Two of the patients who had a pancreatic tumor which involved the gastroduodenal artery developed significant gastrointestinal bleeding following the procedure. However both were managed endoscopically with transfusion, without the need for surgery[30].

A significant drawback of the early PDT treatments was that patients had to spend several days in subdued lighting following the treatment to prevent complications from skin necrosis. However, newer photosensitisers with a shorter drug-light interval and faster drug elimination time have been developed (*e.g.,* verteporfrin) and have been shown in preclinical and early clinical studies to have a similar efficacy and safety profile to mTHPC[33]. A Phase I study by our group evaluated verteporfin-mediated PDT in 15 patients with unresectable locally advanced pancreatic cancer (Vertpac-01) (Table 5)[31,32]. The study was designed in 2 parts: the first 13 patients were treated with a single-fibre, with the following 2 patients being treated with light from multiple fibers. A predictable zone of necrosis surrounding the fibers was achieved. No instances of photosensitivity were reported and only one patient developed cholangitis. Patient went on to receive palliative gemcitabine chemotherapy 28 d after ablation.

YAG Laser

The neodymium-doped yttrium aluminium garnet (Nd: YAG) laser has been used to ablate pancreatic tumours in animal models[34]. A well demarcated area of necrosis and no complications were achieved, suggesting the potential for this therapy, but to date there have been no clinical studies.

NON-THERMAL, NON-LASER METHODS OF ABLATION

Many of the studies of thermal and light ablation techniques in locally advanced and metastatic PDAC have suggested that cytoreduction may improve survival. However in the initial clinical studies some of the techniques were associated with unacceptably high rates of complications. This has led to a search for non-thermal alternative ablative therapies for use in PDAC.

High-intensity focused ultrasound

High intensity focused ultrasound (HIFU) therapy is a non-invasive method of ablation. Ultrasound energy from an extracorporeal source is focused on the pancreatic tumour to induce thermal denaturation of tissue without affecting surrounding organs[35]. Multiple non-randomised studies and case series, largely from Asia, have reported preliminary clinical experiences of using HIFU in PDAC. They have demonstrated that the technique is able to achieve tumour necrosis with relatively few side effects (Table 6). Recently a HIFU transducer has been designed which can be attached to an EUS scope to deliver HIFU locally to pancreatic tumours, thus preventing occasional burns to the skin. Initial animal studies have demonstrated that it can successfully abate the normal pancreas and liver[36].

Irreversible electroporation

NanoKnife® (Angiodynamics, Inc., NY, United States) or irreversible electroporation (IRE) is an emerging non-thermal ablative technique which uses electrodes, placed in the tumour, to deliver up to 3 kV of direct current. This induces the formation of nanoscale pores within the cell membrane of the targeted tissue, which irreversibly damages the cell's homeostatic mechanism, causing apoptosis. The United States Food and Drug Administration have recently approved the technique for use in the pancreas.

One of the major advantages of this technique is that it can be used in tumours that are in close proximity to peri-pancreatic vessels without risk of vascular trauma. The largest series of percutaneous IRE in PDAC includes 14 patients who had unresectable tumours and were not candidates for, or were intolerant of standard therapy[37]. The procedure was performed under general anaesthesia with complete muscle paralysis. Two patients

Table 6 Studies of high intensity focused ultrasound in pancreatic ductal adenocarcinoma

Study	*n*	Study	Outcome and survival	Complications
Wang *et al*[59] (non-English article)	15	HIFU monotherapy in late stage PDAC	Pain relief: 13/13 (100%)	Mild abdominal pain (2/15)
Xie *et al*[60] (non-English article)	41	HIFU alone *vs* HIFU + gemcitabine in locally advanced PDAC	Pain relief: HIFU (66.7%), HIFU + gemcitabine (76.6%)	None
Xu *et al*[61] (non-English article)	37	HIFU monotherapy in advanced PDAC	Pain relief: 24/30 (80%)	None
Yuan *et al*[62] (non-English article)	40	HIFU monotherapy	Pain relief: 32/40 (80%)	None
Wu *et al*[63]	8	HIFU in advanced PDAC	Median survival: 11.25 mo Pain relief: 8/8	None
Xiong *et al*[64]	89	HIFU in unresectable PDAC	Median survival: 26.0 mo (stage Ⅱ), 11.2 mo (stage Ⅲ) and 5.4 mo (stage Ⅳ)	Superficial skin burns (3.4%), subcutaneous fat sclerosis (6.7%), asymptomatic pseudocyst (1.1%)
Zhao *et al*[65]	37	Phase Ⅱ study of gemcitabine + HIFU in locally advanced PDAC	Overall survival: 12.6 mo (95%CI: 10.2-15.0 mo) Pain relief: 78.6%	16.2% experienced grade 3 or 4 neutropenia, 5.4% developed grade 3 thrombocytopenia, 8% had nausea vomiting
Orsi *et al*[66]	6	HIFU in unresectable PDAC	Pain relief: 6/6 (100%)	Portal vein thrombosis (1/6)
Sung *et al*[67]	46	Stage Ⅲ or Ⅳ PDAC	Median survival: 12.4 mo. Overall survival at 12 mo was 30.4%	Minor complications (abdominal pain, fever and nausea): 57.1% (28/29) Major complications (pancreaticoduodenal fistula, gastric ulcer or skin burns): 10.2% (5/49)
Wang *et al*[68]	40	Advanced PDAC	Median overall survival: 10 mo (stage Ⅲ) and 6 mo (stage Ⅳ). Pain relief: 35/40 (87.5%)	None
Lee *et al*[69]	12	HIFU monotherapy in unresectable PDAC (3/12 received chemotherapy)	Median overall survival for those receiving HIFU alone (9/12 patients): 10.3 mo	Pancreatitis: 1/12
Li *et al*[70]	25	Unresectable PDAC	Median overall survival: 10 mo. 42% survived more than 1 year. Performance status and pain levels improved: 23/25	1st degree skin burn: 12% Mortality: 0%
Wang *et al*[71]	224	Advanced PDAC	Not reported	Abdominal distension, anorexia and nausea: 10/ 224 (4.5%). Asymptomatic vertebral injury: 2/224
Gao *et al*[72]	39	Locally advanced PDAC	Pain relief: 79.5% Median overall survival: 11 mo. 30.8% survived more than one year	None

HIFU: High intensity focused ultrasound; PDAC: Pancreatic ductal adenocarcinoma.

subsequently underwent surgery after IRE and both had margin-negative resections; both remain disease-free after 11 and 14 mo, respectively. Complications included spontaneous pneumothorax during anaesthesia (n = 1) and pancreatitis (n = 1); both patients recovered completely. No deaths were related to the procedure but the three patients with metastatic disease subsequently died from disease progression.

COMBINING ABLATIVE THERAPIES WITH BILIARY STENTING

Tumours of the head of the pancreas commonly cause distal biliary obstruction, which is managed in most cases by an endoscopically inserted self-expanding metal stent (SEMS). However due to tumour ingrowth SEMS are associated with a shorter patency time than bypass surgery. Hence there has been a growth in interest in using ablative therapies such as PDT or RFA to prolong stent patency or to unblock a SEMS, which is already *in situ*. Randomised studies comparing PDT with biliary stenting to stenting alone have had conflicting results. Initial studies reported prolonged stent patency and improved survival after PDT[38,39]. However, a recent UK phase Ⅲ study closed early as overall survival was longer in those treated with stenting alone[40]. The use of RFA in combination with SEMS placement has been reported in two small studies to date (Table 3). The investigators showed that the median bile duct diameter increased following endobiliary RFA and that 86% (19/22) of the SEMS were patent at 90 d[41,42]. Emerging evidence also suggests that endobiliary RFA may confer some early survival benefit in patients with malignant biliary obstruction independent of stent blockage and chemotherapy[41]. Occasionally centres have used RFA alone to achieve biliary drainage but results of on-going randomised controlled trials are awaited for validation of this technique[43]. Current guidance from the National Institute for Health and Care Excellence in the United Kingdom recommends

that this treatment should only be carried out in specialist centres in the context of clinical trials[44].

ENDOSCOPIC ULTRASOUND GUIDED NON-ABLATIVE LOCAL THERAPIES

Systemic chemotherapy agents are often associated with significant side effects, which can result in patients having to stop therapy or undergo dose reduction. Several groups have therefore explored using local anti-tumour agents in PDAC. The outcomes are summarised in Table 2.

PREMALIGNANT LESIONS OF THE PANCREAS

Some investigators have used similar ablative methods in PDAC to ablate premalignant solid and cystic lesions of the pancreas. Cystic lesions of the pancreas are an increasingly common clinical finding and some possess premalignant potential; longterm surveillance or surgery or pancreatic surgery is therefore recommended in accordance with international guidance[45]. Given the morbidity of surgery and uncertainties of surveillance for essentially benign disease, minimally invasive ablative therapies are increasingly becoming an attractive alternative treatment.

An EUS-guided injection of alcohol has been reported to have reasonable efficacy for achieving complete ablation of pancreatic cystic tumours (35%-62%). However, total cyst ablation was rare in septated cysts and the technique was associated with complications (pain and pancreatitis) in between 4%-20% of cases[46,47]. Occasional case reports have described using EUS guided alcohol injection to successfully ablate hepatic metastases[48] and pancreatic gastrointestinal stromal tumours[49]. Small case series have demonstrated EUS guided RFA can also be used safely for this indication[21]. Further validation will come from larger Phase Ⅱ studies.

CONCLUSION

Ablative therapies for unresectable pancreatic cancer are an attractive emerging therapy. All studies demonstrated that ablation is feasible and reproducible. Many of the early concerns that surrounded safety have been addressed with device development and modification of technique. Long-term survival data for many of the techniques is absent currently. Ultimately large prospective randomised studies will be required to assess the efficacy of these techniques and define their position in future treatment algorithms for the management of locally advanced pancreatic cancer.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Role of abnormal lipid metabolism in development, progression, diagnosis and therapy of pancreatic cancer

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Abstract

There is growing evidence that metabolic alterations play an important role in cancer development and progression. The metabolism of cancer cells is reprogrammed in order to support their rapid proliferation. Elevated fatty acid synthesis is one of the most important aberrations of cancer cell metabolism. An enhancement of fatty acids synthesis is required both for carcinogenesis and cancer cell survival, as inhibition of key lipogenic enzymes slows down the growth of tumor cells and impairs their survival. Based on the data that serum fatty acid synthase (FASN), also known as oncoantigen 519, is elevated in patients with certain types of cancer, its serum level was proposed as a marker of neoplasia. This review aims to demonstrate the changes in lipid metabolism and other metabolic processes associated with lipid metabolism in pancreatic ductal adenocarcinoma (PDAC), the most common pancreatic neoplasm, characterized by high mortality. We also addressed the influence of some oncogenic factors and tumor suppressors on pancreatic cancer cell metabolism. Additionally the review discusses the potential role of elevated lipid synthesis in diagnosis and treatment of pancreatic cancer. In particular, FASN is a viable candidate for indicator of pathologic state, marker of neoplasia, as well as, pharmacological treatment target in pancreatic cancer. Recent research showed that, in addition to lipogenesis, certain cancer cells can use fatty acids from circulation, derived from diet (chylomicrons), synthesized in liver, or released from adipose tissue for their growth. Thus, the interactions between *de novo* lipogenesis and uptake of fatty acids from circulation by PDAC cells require further investigation.

Key words: Pancreatic cancer; Lipid metabolism; Fatty acid synthase; Monounsaturated fatty acids; Farnesylation; Hypoxia inducible factor 1α; Cyclooxygenase-2; Oncogenes; Tumor suppressors; Lipogenic enzymes inhibitors

Core tip: Metabolic alterations associated with mutation in oncogenes and tumor suppressor genes play an important role in cancer development and progression. One of the most important aberrations of metabolism in cancer cells is an elevated synthesis of lipids, which are building blocks for cell membrane formation during cell proliferation and signalling molecules. This review aims to demonstrate the changes in lipid metabolism in pancreatic ductal adenocarcinoma, the most common pancreatic neoplasm, with very high mortality. The potential role of elevated lipid synthesis in diagnosis, prognosis and therapy of pancreatic cancer is also discussed.

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INTRODUCTION

Cancer development is generally attributed to the accumulation of genetic alterations, which leads to activation of cellular oncogenes and inactivation of tumor suppressor genes. Apart from mutations, epigenetic modulation, numerical and structural abnormalities in chromosomes, and aneuploidy are commonly observed in cancer cells, and may play a critical role in tumorigenesis[1]. In addition, carcinogenesis involves significant changes in cellular metabolism, especially in carbohydrate, lipid, nucleic acid, and amino acid metabolism (Figure 1).

The metabolism of cancer cells is reprogrammed in order to support their rapid proliferation. Nowadays, metabolic alteration, also referred to as metabolic transformation, should be added to six classic hallmarks of cancer cells proposed by Hanahan and Weinberg[2], Tennant *et al*[3] and illustrated on Figure 2. Over eight decades ago, Warburg revealed that an elevated rate of glycolysis under aerobic conditions, a phenomenon commonly known as the Warburg effect, is a distinctive feature of many human and animal tumors[4]. In the majority of cancers, glucose is converted mostly to lactate, and, therefore, only 2 moles of ATP per 1 mole of glucose are synthesized. In contrast, most non-cancer cells containing mitochondria, produce CO_2 and H_2O from glucose, and 38 moles of ATP are synthesized per 1 mole of glucose, under aerobic conditions.

Over the last two decades, several authors reported overexpression of genes encoding lipogenic enzymes in many human cancers (Table 1)[5-12]. This phenomenon is usually associated with an increased glucose carbon incorporation into lipids[13-16]. The possible pathways for the conversion of glucose into phospholipids and cholesterol, required for membrane formation in cancer cells, are illustrated on Figure 1. Pyruvate formed from glucose during active aerobic glycolysis, is either converted to lactate by lactate dehydrogenase (LDH), or can enter into mitochondria, where it is decarboxylated to acetyl-CoA by pyruvate dehydrogenase (PDH). Then, by means of reactions of citrate synthase (CS), present in mitochondria, and ATP citrate lyase (ACLY), present in cytosol, cytosolic acetyl-CoA, a key substrate for lipid biosynthesis is formed (Figure 1). Elevated activities of both enzymes (CS and ACLY) are observed in some malignancies, and the inhibition of ACLY is known to lead to cessation of tumor growth[17-21]. Interestingly, some tumors display a diminished flux of glucose carbon through PDH-catalyzed reaction, due to increased PDHK (pyruvate dehydrogenase kinase) activity, under the influence either hypoxia or oncogenic factors. This points to the possible use of carbon source other than glucose, for lipid synthesis[22-25].

Through conversion to fructose 6-phosphate, glucose also serves as a substrate for hexosamine phosphate synthesis (according to reaction: fructose 6-phosphate + glutamine → glucosamine 6 phosphate + glutamate), required for biosynthesis of glycoproteins and glycosaminoglycans. Glucose may also be converted to pentose phosphate on pentose phosphate pathway (PPP), and then to phosphoribosyl pyrophosphate (PRPP), a precursor of purine and pyrimidine nucleotides necessary for DNA synthesis (Figure 1). PPP generates NADPH, which is required for many processes, including lipid biosynthesis (Figure 1). The activity of glucose 6-phosphate dehydrogenase (G6PDH), a rate limiting enzyme of PPP, is elevated in certain cancers, including human pancreatic cancer (PC)[19,26]. Glutamine for hexosamine and nucleotide synthesis may originate from citrate produced in mitochondria. Citrate is converted by Krebs cycle to 2-oxoglutarate, a precursor of glutamate (Figure 1), and later to glutamine. However, glutamine is not synthesized on that pathway in many cancer cells, but is rather taken up from the circulation, where it is one of the most abundant amino acids[27].

Glucose and glutamine are two main sources of energy and carbon for most cancer cells[28-30]. Some data suggest that glucose accounts mainly for lipid, purine, and pyrimidine nucleotide synthesis, whereas glutamine is contributing to: (1) anaplerotic re-feeding of Krebs cycle; (2) amino acid synthesis; and (3) providing nitrogen necessary for purine and pyrimidine nucleotide synthesis[14], however, there is also evidence of glutamine participation (as carbon donor) in lipid biosynthesis[31]. High expression of glutaminase-encoding gene was revealed during the S phase of the cell cycle in some cancer cell lines (*i.e.* HeLa cells), along with the low expression in G_2/M phase[32]. Upon cellular uptake, glutamine is transported to mitochondria, and then converted to ammonia and glutamate by mitochondrial glutaminase. Then glutamate is deaminated to 2-oxoglutarate by glutamate dehydrogenase. In mitochondria, 2-oxoglutarate is further metabolized by Krebs cycle to malate (Figure 3). Part of the malate is released to cytosol, converted to pyruvate by NADP-linked malic enzyme (ME), and, finally, to lactate by LDH, similarly to pyruvate formed from glucose during glycolysis (Figure 3). The conversion of glutamine to lactate is called glutaminolysis analogically to glycolysis (Figure 3). The increased synthesis of lactic acid by cancer cells leads to the decrease in pH of tumor microenvironment, which promotes angiogenesis, invasion, and metastasis, and suppresses the anticancer immune response through diminished cytotoxic T-cell function[33] (Figure 3).

In a variety of tumors, pyruvate formed during active glutaminolysis is converted into acetyl-CoA by PDH (instead of being converted to lactate by LDH), and later to citrate, supplying carbons for lipid synthesis (Figure 3)[34,35]. Conversion of glutamine to citrate may be also the result of reductive carboxylation of 2-oxoglutarate

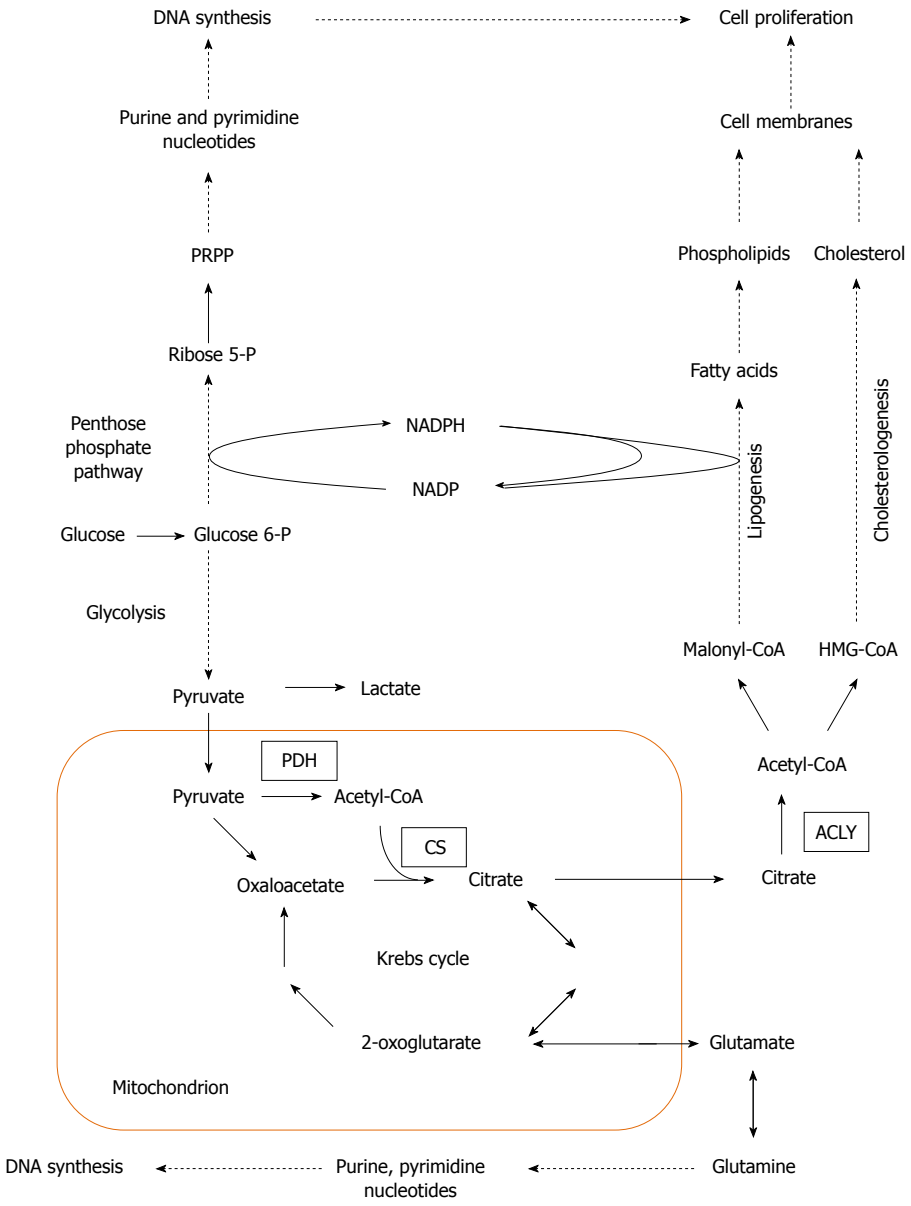

Figure 1 Cellular metabolism of cancer cells-association with cell proliferation. Solid arrows represent single reactions; dotted arrows represent processes including numerous reactions. PRPP: Phosphoribosyl pyrophosphate; Ribose 5-P: Ribose 5-phosphate; Glucose 6-P: Glucose 6-phosphate; PDH: Pyruvate dehydrogenase; CS: Citrate synthase; ACLY: ATP citrate lyase; HMG-CoA: 3-hydroxy-3-methylglutaryl-coenzyme A.

derived from glutamine, catalyzed by two isoforms of $NADP^+$-dependent isocitrate dehydrogenase - mitochondrial (IDH2), and/or cytosolic (IDH1) (Figure 1)[36-40]. In some cancer cell lines 10%-25% of fatty acids carbons are derived from glutamine under normoxia, and up to 80% under hypoxia[14,36,37]. Wise *et al*[38] suggest that IDH2 is mainly contributing to conversion of glutamine to lipids. However, other data show that in A549 (adenocarcinoma of human alveolar basal epithelial cells), and in renal carcinoma cells (RCC) cell lines IDH1 is more important[36]. In melanoma or osteosarcoma cell lines both IDH isoforms equally participate in 2-oxoglutarate reduction[37,40].

Continuous loss of citrate from mitochondria to cytosol requires replenishment of Krebs cycle intermediates. Glutamine serves as a key substrate for Krebs cycle intermediates in many cancer cells, and is critical for cell proliferation. A proliferating cell dies upon glutamine (but not glucose) withdrawal from the medium[41].

Fatty acid (FA) biosynthesis remains at a low level in most non-cancerogenic tissues, except liver and adipose tissue. The two latter lipogenic tissues convert the excess of carbohydrates to triacylglycerols[42-49]. Conversely FAs synthesized in cancer cells are esterified mainly to phospholipids required for membrane formation, which promotes cellular replication (Figure 1). Overall, coordinated enhancement of glucose, lipid, and amino acid metabolism, leading to increased synthesis of membrane lipids, nucleotides, and amino acids supports rapid proliferation of cancer cells (Figure 1).

Proliferation and metabolism of cancer cells share common regulatory pathways[50-53]. MYC, proto-oncogene and major regulator of transcription in growing cells, controls several metabolic processes such as: (1)

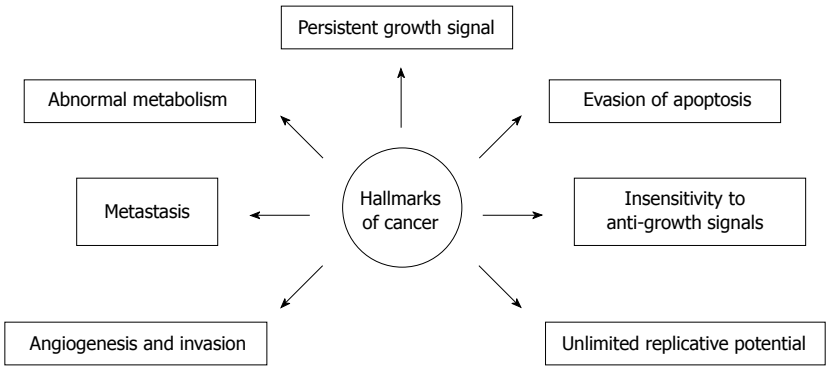

Figure 2 Hallmarks of cancer.

Table 1 Overexpression of lipogenic enzymes in human tumors

Enzyme name	Neoplasm type	Experimental model	Ref.
Fatty acid synthase	Pancreatic cancer	Human tumor tissue, cell line	[96,104,105]
(FASN)	Breast carcinoma	Human tumor tissue	[5,9,166]
	Prostate cancer	Human tumor tissue	[167]
	Melanoma	Human tumor tissue	[168]
	Nephroblastoma	Human tumor tissue	[169]
	Renal cancer	Cell line	[170]
	Endometrial carcinoma	Human tumor tissue	[12,171]
	Colon cancer	Human tumor tissue	[11,172]
	Ovarian neoplasms squamous cell	Human tumor tissue	[10,173]
	Carcinoma of the lung head and neck squamous	Human tumor tissue	[174]
	Cell carcinoma squamous cell	Human tumor tissue	[175]
	Carcinoma of the tongue	Human tumor tissue	[176]
ATP citrate lyase	Small cell lung cancer	Cell line	[251]
(ACLY)	Bladder cancer	Human tumor tissue	[7]
	Breast cancer	Cell line	[252]
	Gastric cancer	Human tumor tissue, cell line	[253]
	Colon cancer	Human tumor tissue	[254]
	Prostate cancer	Human tumor tissue	[254]
	Hepatocellular carcinoma	Human tumor tissue	[255]
Acetyl-CoA carboxylase	Prostate cancer	Human tumor tissue	[6]
(ACCA)	Hepatocellular carcinoma	Human tumor tissue	[255]
	Breast carcinoma	Human tumor tissue	[256]
Stearoyl-CoA desaturase	Pancreatic cancer	SCD1 indices in patients serum	[128]
(SCD1)	Clear cell renal cell carcinoma	Human tumor tissue	[200]
Acetyl-CoA synthetase	Colon adenocarcinoma	Human tumor tissue	[257]
(ACS)	Malignant glioma	Cell line	[258]
Citrate synthase	Pancreatic cancer	Human tumor tissue	[19]
(CS)	Renal cell carcinoma	Human tumor tissue	[20]

glycolysis and glutaminolysis; (2) nucleotide biosynthesis; and (3) lipid biosynthesis, and mitochondrial biogenesis[53]. Furthermore, MYC stimulates glutamine uptake and metabolism[54,55]. Tumor suppressor protein, p53, is involved in regulation of bioenergetic homeostasis and lipid metabolism in both normal and cancer cells[51,56-58]. p53 induces the expression mitochondrial glutaminase-encoding gene, increasing energy production from glutaminolysis[59,60]. Mutant p53 increases lipid synthesis, *via* sterol regulatory element-binding protein 1c (SREBP1c), and promotes ovarian cancer metastasis[52]. Certain oncoproteins such as: Akt, Ras, and Src, also stimulate glycolysis in transformed cells[50]. Regulation of glutamine metabolism by Rho GTPases and Ras was also proposed[61]. The oncogenes and tumor suppressor genes whose products participate in regulation of carbohydrate, lipid, nucleotide and amino acid metabolism are presented in Table 2.

Also mutations of some genes can contribute to abnormal cellular metabolism, which in turn can affect oncogenic signaling pathways. For example mutation in gene encoding IDH1/2 is associated with deregulation of cellular metabolism, especially in glioma cells[62]. In glioma IDH1/2 mutations are responsible for conversion of 2-oxoglutarate to 2-hydroxyglutarate, which, by inhibition of 2-oxoglutarate-dependent dioxygenases, affects: (1) proto-oncogene expression; (2) DNA and histone modification; and (3) alteration of extracellular matrix proteins (due to inhibition of collagen hydroxylation)[62]. This paper reviews the possible role of lipid metabolism in human cancers, particularly in PC biology, prognosis, and treatment.

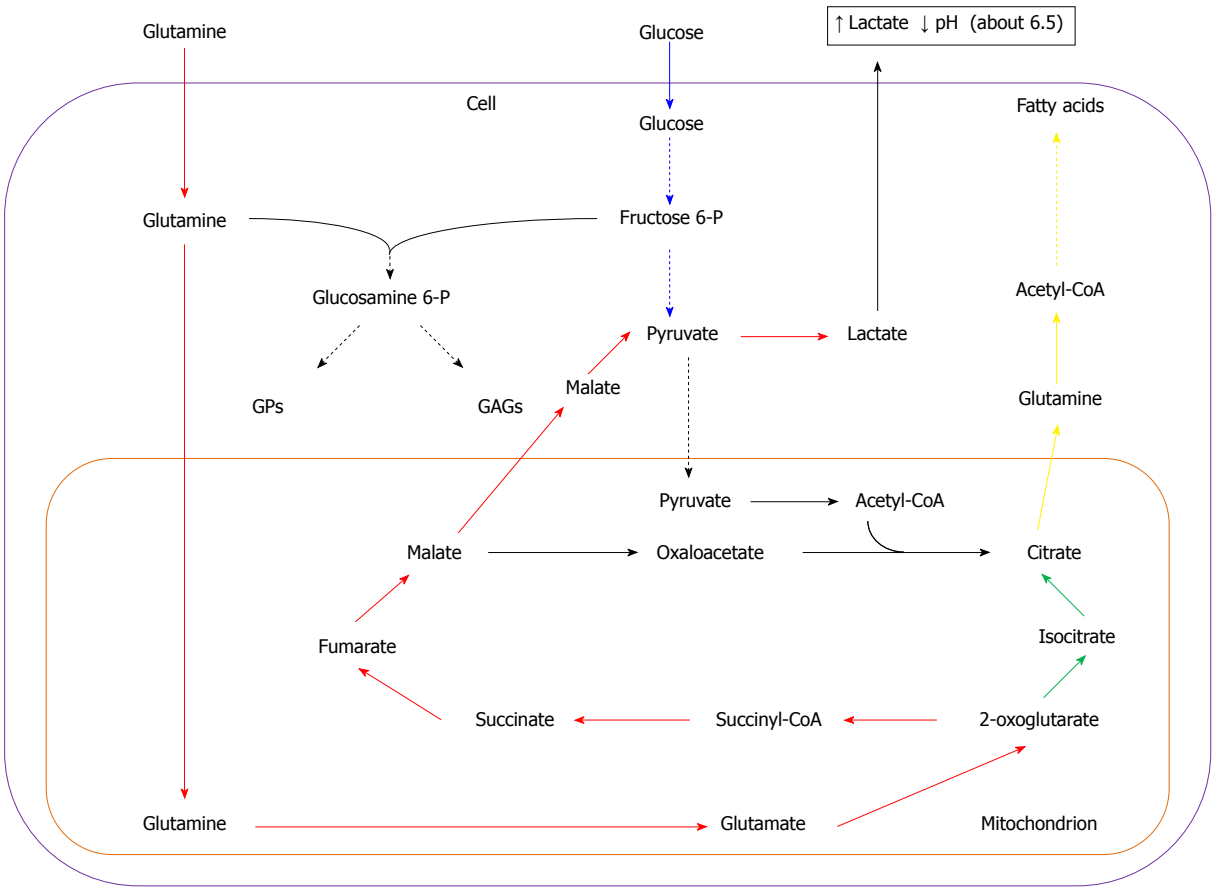

Figure 3 Glutamine metabolism of cancer cells. Red arrows represent glutaminolysis; green arrows represent "reversed Krebs cycle" reactions; blue arrows represent glycolysis; yellow arrows represent lipogenesis. Solid arrows represent single reactions; dotted arrows represent processes including numerous reactions. Fructose 6-P: Fructose 6-phosphate; GAGs: Glycosaminoglycans; GPs: Glycoproteins.

ABNORMAL LIPID METABOLISM IN PANCREATIC CANCER

Pancreatic ductal adenocarcinoma (PDAC) is the most common pancreatic neoplasm, comprising approximately 90% of all pancreatic malignancies, and the eight leading cause of cancer-associated death in the world[63]. The 5-year survival rate of PDAC patients is approximately about 5%[64]. Surgery is the primary treatment modality and the only available chance for recovery, however only approximately 10% of patients are eligible for surgical treatment. Other therapies have proven ineffective thus far.

Similar to other cancers, both activation of oncogenes and inactivation of tumor suppressor genes play key role in PDAC pathogenesis. The most frequent genetic alterations documented in PCs, including PDAC, are presented in Table 3. Other pancreatic tumors show different aberrations (Table 4).

In addition to genetic and epigenetic alterations, development of PC involves significant alterations of cellular metabolism, supporting rapid proliferation of cancer cells. Reduced vascularity, leading to poor perfusion is characteristic for PC. This results in low availability of oxygen and nutrients[65,66]. The presence of hypoxia corresponds to highly aggressive character of PCs[67].

Oxygen deprivation of both non-cancer and cancer cells leads to the stabilization of hypoxia inducible factor 1α (HIF-1α), which dimerizes with HIF-1β, transfers into nucleus and binds with hypoxia-responsive elements present in DNA (Figure 4). This counteract the deleterious impact of decreased oxygen availability[68]. High level of HIF-1α is associated with increased glucose consumption due to activation of glucose transporter 1 (GLUT1), and glycolysis, especially hexokinase (1 and 2), and LDH[69-73] (Figure 4). Overexpression of HIF-1α in human PC cells makes this malignancy similar to other cancers[74]. Interestingly, the expression of HIF-1α in the hypoxic part of pancreatic tumor is at the same level as in its well-oxygenated fragments[75]. Some data indicate that phosphorylation of HIF-1α weakens the interaction of this protein with von Hippel-Lindau tumor suppressor (VHL) which normally stimulates degradation of HIF-1α during normoxia (Figure 4). The phosphorylation result from activation of MAPK or other protein kinase (putatively AKT) in cancer cells[76]. Both kinases are downstream effectors in various signaling pathways, including KRAS pathway. Continuous KRAS signaling and downstream activation of MAPK and AKT results from the mutation of KRAS (observed in 90% of PDACs), or can be stimulated by epidermal growth factor (EGF), prostaglandin E_2 (PGE_2), and some oxidants[73,77,78].

Table 2 Oncogenes and tumor suppressor genes, whose products participate in regulation of cancer cells metabolism

Oncogene/tumor suppressor	Metabolic pathway	Enzyme	Ref.
MYC	Glucose transport	GLUT1	[53-55]
	Glycolysis	Hexokinase 2	
		Phosphohexose isomerase	
		Phosphofructokinase 1	
		Aldolase A	
		3-phosphoglyceraldehyde dehydrogenase	
		Phosphoglycerate kinase	
		Phosphoglycerate mutase	
		Enolase 1	
		Pyruvate kinase 2	
		Lactate dehydrogenase A	
	Regulation of PDH	Pyruvate dehydrogenase kinase 1	
	Glutamine transport	Glutamine transporters ASCT2 and SN2	
	Glutaminolysis	Glutaminase 1	
	Serine hydroxymethyltransferase		
	Pyrimidine synthesis		
	Aminoacids metabolism	CAD	
		Ornithine decarboxylase	
	Lipogenesis	Fatty acid synthase	
p53	Glucose transport	GLUT1	[51,56-60]
	Glycolysis	Hexokinase 2	
		Fructose-2,6-bisphosphatase	
		Phosphoglycerate mutase	
	Oxidative phosphorylation	Cyrochrome c oxidase	
	Glutaminolysis	Glutaminase 2	
	Pentose Phosphate Pathway	Glucose-6-phosphate dehydrogenase	
	Regulation of PDH	Pyruvate dehydrogenase kinase 1	
	Krebs cycle	Aconitase	
KRAS	Glucose transport	GLUT1	[61,73,94]
	Glycolysis	Hexokinase 2	
		Phosphofructokinase 1	
		Lactate dehydrogenase A	
	Pentose phosphate pathway	Transketolase	
	Hexosamine synthesis	Phosphohexose aminotransferase	
	Glutaminolysis	Glutamate dehydrogenase	
		Aspartate transaminase	
Akt/PTEN	Glucose transport	GLUT1	[50,113-115]
	Lipogenesis	FASN	

PI3K/Akt signaling pathway leads to overexpression of HIF-1α, and directly participates in glucose transport and metabolism by regulating GLUT1 gene expression in PC cells, especially when the function of PTEN, tumor suppressor inhibiting PI3K/AKT pathway, is lost[79-81]. Another oncogene, MYC, interacts with KRAS and HIF-1α in PDAC metabolic switch. MYC response elements are present in most glycolytic genes, thus, allowing MYC protein to regulate glucose metabolism[73,82]. Some data suggest that activation of HIF-1α, leading to metabolic reprogramming of pancreatic cells during normoxia, is also controlled by β-adrenergic receptors through the transactivation of epidermal growth factor receptor (EGFR, requiring PKA activity), and further activation of AKT[83]. Also insulin, causing activation of PI3K/AKT and MAPK pathways, can be potential stimulator of HIF-1α activity acting independently of oxygen availability[84].

Mucin 1 (MUC1), a transmembrane protein involved in stabilization of HIF-1α is one of the newly discovered activators of HIF-1α in PC. Directly interacting with HIF-1α and DNA, MUC1 induces expression of glycolytic genes[85]. High activity of MUC1 is correlated with intensive growth and metastasis of pancreatic tumors[86,87]. HIF-1α is coexpressed with Nupr1 (also known as p8 or Com, *i.e.* candidate of metastasis) in human PDAC[88]. Nupr 1 is a chromatin protein, structurally related to the high-mobility group (HMG) protein, it interacts with several other proteins in the regulation of cell cycle, apoptosis, autophagy, and gene transcription[89]. It is responsible for increased resistance of stress-exposed PDAC cells[90] and supposedly interacts and amplify the KRAS signaling in cancer cells, in order to overcome the activity of some tumor suppressors (such as p16) action[91].

The data presented above suggest that several proteins (mainly products of proto-oncogens or tumor suppressor genes) might affect conversion of glucose to pyruvate in PDAC cells.

Most of the pyruvate formed as a result of increased glycolysis in PDAC cells, is metabolized to lactate, some pyruvate is used to citrate, and further to FAs biosynthesis[92,93]. Accordingly, the activity of CS, one of the crucial enzymes involved in pyruvate to FA conversion (Figure 1), is elevated in PC[19,20]. Thus, it is likely that citrate is synthesized from glucose in PC cells, although glutamine seems to play an important role as well.

Table 3 Oncogenes and tumor suppressor genes whose products alter the metabolism of pancreatic cancer cells

Gene	Protein	Mechanism of alteration in PDAC	Regulated processes in PDAC	Alteration in PDAC	Ref.
Oncogenes					
KRAS	KRAS	Point mutations	Cell proliferation and survival, motility, glucose transport, glycolysis, hexosamine synthesis, nonoxidative pentose phosphate pathway arm, glutaminolysis	> 95%	[73,94,259-261]
AKT	AKT	Mutations, amplification	Signal transduction, lipogenesis, glucose transport	10%-20%	[73,79-81,262-264]
c-erbB2	HER2	Overexpression amplification	Proliferation, differentiation, survival	20%-80%	[265-268]
Myc	MYC	Amplification overexpression	Glycolysis, glutaminolysis, PDH inhibition	70%	[55,73,82,94,269]
Tumor suppressor genes					
TP53	p53	Mutation and second allele deletion	Cell cycle, apoptosis, DNA repair, glucose transport, glycolysis, lipogenesis, ppp oxidative arm, glutaminolysis	50%-80%	[270-273]
Smad4/DPC4	SMAD4	Homozygous deletion, mutation and second allele deletion	Cell cycle, TGF-β signaling	55%	[274-276]
STK/LKB1	LKB1	Homozygous deletion, mutation and second allele deletion	Apoptosis, lipogenesis, energy production, protein synthesis	5%	[277-279]
CDKN2A/p16	p16	Homozygous deletion, mutation, hypermethylation	Cell cycle	95%	[280-282]
PTEN	PTEN	Hypermethylation, inhibition by miRNA	PI3K/AKT signaling pathway	30%-70%	[79,283,284]

PDAC: Pancreatic ductal adenocarcinoma.

Table 4 Most common genetic alterations observed in different types of human pancreatic cancers

Type of pancreatic cancer	Gene affected	Ref.
Pancreatic ductal adenocarcinoma (PDAC) (90% of all pancreatic cancers)	KRAS, AKT, MYC, TP53, SMAD4, CDKN2A, PTEN	[55,63,64,73,78,79,94,269,284-287]
Acinar cell carcinoma (ACCA) (< 1% of all pancreatic cancers)	APC/β-catenin (CTNNB1), BRCA2, BCL10	[288-290]
Adenosquamous carcinoma (ASC) (< 1% of all pancreatic cancers)	TP53, CDKN2A, KRAS, E-cadherin,	[291,292]
Intraductal papillary mucinous neoplasm (IPNM) (1%-3% of all pancreatic cancers)	GNAS, KRAS, RNF4, STK11/LKB1, MUC1, MUC2, hTERT, COX2, Shh	[278,293,294]
Mucinous cystic neoplasm (MCN) (< 1% of all pancreatic cancers)	KRAS, RNF4, TP53, CDKN2A	[295]
Serous cystadenoma (SCN) (< 1% of all pancreatic cancers)	VHL	[296]
Solid-pseudopapillary neoplasm (SPN) (1%-2% of all pancreatic cancers)	APC/β-catenin (CTNNB1), E-cadherin	[297,298]
Pancreatic neuroendocrine tumors (PanNET) (2%-5% of all pancreatic cancers)	DAXX, ATRX, MEN1, TSC2, PTEN, PI3KCA, CHGA, CHGB, mTOR	[299-302]

Son *et al*[94] suggested that KRAS directs glutamine carbons to Krebs cycle in PC cells, to export them to cytosol for cytosolic ME reaction. This results in the generation of NADPH, which is used for lipid biosynthesis and for redox state control. Deprivation of glutamine or inhibition of glutaminase activity are reflected by decreased production of ATP and higher levels of reactive oxygen species (ROS). Glutamine may also supply OAA, condensed with acetyl-CoA, to citrate synthesis, or be involved in citrate formation through reductive carboxylation of 2-oxoglutarate catalyzed by reverse IDH reaction. Although the involvement of glutamine was documented in some malignancies, its role in PC cells is still not completely understood[14,36-40,95]. Nevertheless, *de novo* biosynthesis of lipids (possibly from glucose and/or glutamine) is elevated in PDAC cells[96-98].

Gemcitabine, herceptin or irinotecan treatment has minimal impact on survival rates in patients with advanced PC[99,100]. In contrast treating PC patient with gemcitabine, α-lipoic acid, and hydroxycitrate yielded promising results[101]. Since hydroxycitrate is an inhibitor of ACLY, the activity of the latter lipogenic enzyme (splitting citrate to acetyl-CoA and OAA in cytosol) is likely elevated in PC cells as well, and, similar to other cancers, plays an important role in the development of this malignancy. The next stage of lipogenesis, leading to biosynthesis of malonyl-CoA (fatty acid synthase substrate), is catalyzed by acetyl-CoA carboxylase (ACCA). Phosphorylation by AMPK, leading to ACCA activity cessation, is one of the crucial stages of lipogenesis regulation in lipogenic tissues[102]. The activity of AMPK in PDAC cells is lower than in normal cells, mostly due to LKB1 tumor suppressor inhibition, leading to increased ACCA activity[103]. Fatty acid synthase (FASN) reaction constitutes the last step in palmitate synthesis. The significant role of FASN in cancer development was established approximately two

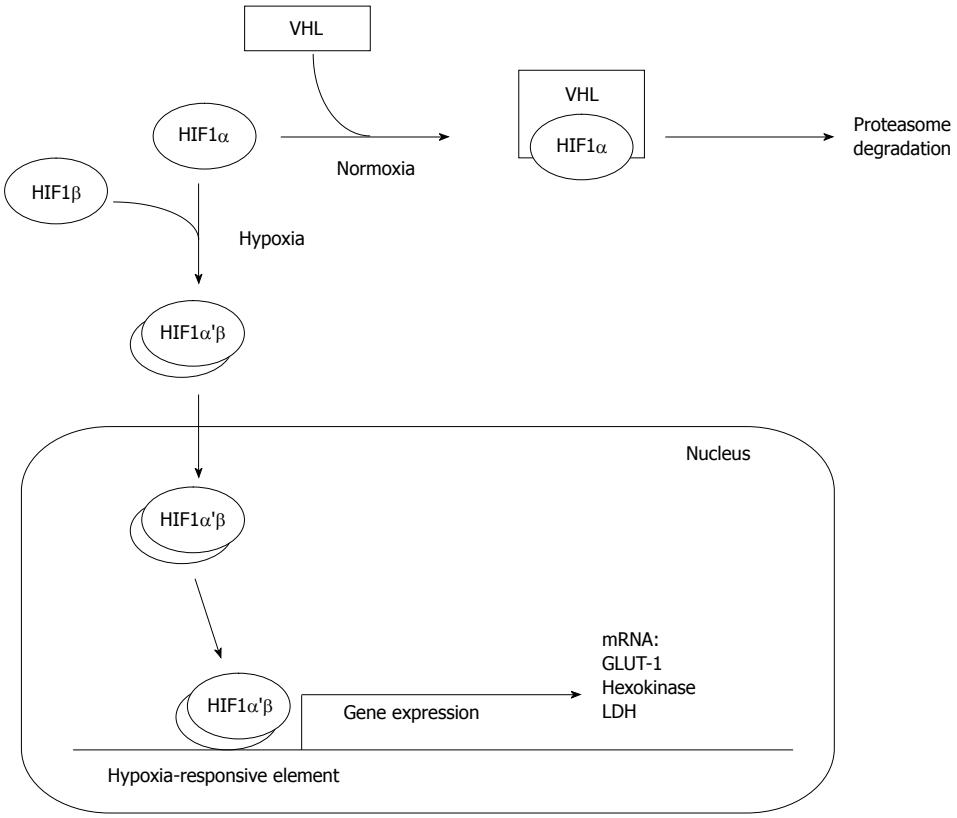

Figure 4 Role of hypoxia inducible factor 1 α'β in pancreatic cancer. HIF1α: Hypoxia inducible factor 1α; HIF1β: Hypoxia inducible factor 1β; VHL: Von Hippel-Lindau tumor suppressor; GLUT-1: Glucose transporter 1; LDH: Lactate dehydrogenase.

decades ago, when the oncogenic antigen-519 (OA-519), a molecular marker, was identified in breast cancer patients[9]. FASN utilizes acetyl-CoA (supplied by ACLY), malonyl-CoA (supplied by ACCA) and NADPH as a reducing equivalent. In the case of PC cells, NADPH is a product of PPP or reaction catalyzed by ME during oxidative decarboxylation of malate formed from glutamine (*i.e.* during glutaminolysis)[94]. FASN is the most extensively studied lipogenic enzyme in PDAC cells. Elevated expression of FASN-encoding gene was documented in human PC[96,104,105] and high level of FASN protein, both in tumor cells and in serum is associated with poor prognosis[96,98]. Furthermore inhibition of FASN activity was revealed to induce apoptosis in several tumors[106-110]. Indeed, FASN is an oncogenic protein and its overexpression in non-transformed human breast epithelial cells, can produce their cancer-like phenotype, in a HER1/2 dependent process[111]. Similar phenomenon was reported in the case of colorectal cancer cells[112]. The expression of *FASN* is strongly induced in hypoxia, by MAPK or PI3K/AKT signaling pathways. This results in activation of SREBP1c transcription factor, which directly binds to FASN promoter (and promoters of other lipogenic genes)[113,114]. Similar effect can be observed in the absence of PTEN tumor suppressor, which normally inhibits PI3K/AKT signaling[114,115]. Moreover, SREBP1c-independent regulation of *FASN*, mediated by HER2 with PI3K or mTOR involvement was observed in breast cancer cells[116]. Furthermore strong acidic environment of breast cancer may promote epigenetic modification of *FASN* promoter, leading to increased expression of this gene[117]. As all those events take place in PC cells, the mechanism of *FASN* regulation in PDAC is probably similar as in the case of other malignancies.

Inhibited activity of FASN (or other lipogenic enzymes) is reflected by decreased tumor growth and may lead to apoptosis of some cancer cells. The inhibition of FASN was revealed to diminish proliferation of osteosarcoma and colorectal cancer cells, through decrease of HER2 activity, leading to down-regulation of PI3K/Akt signaling pathway[112,118]. Induction of apoptosis is likely to result from elevated concentration of malonyl-CoA, that is reflected by decreased oxidation of FA and increased ceramide concentration. Ceramide is a well-known activator of apoptosis, and its enhanced biosynthesis (along with inhibited ceramidase activity) leads to the death of PC cells[106,119]. Furthermore the altered composition of FAs in phospholipid structure (predominance of polyunsaturated acids over saturated and monounsaturated acids) increases the oxidative stress yielding the same result[120].

Glycolytic synthesis of ATP seems the most important pathway in hypoxic cancer cells. In the cases of normoxia, glucose is rather directed to PPP for NADPH and pentose synthesis, and KRAS acts as the main controlling factor supporting tumor cell proliferation[121,122]. Both oxidative and non-oxidative phases of PPP are upregulated in PC cells. The non-oxidative phase is upregulated by KRAS[73,123], whereas G6PDH activity (main

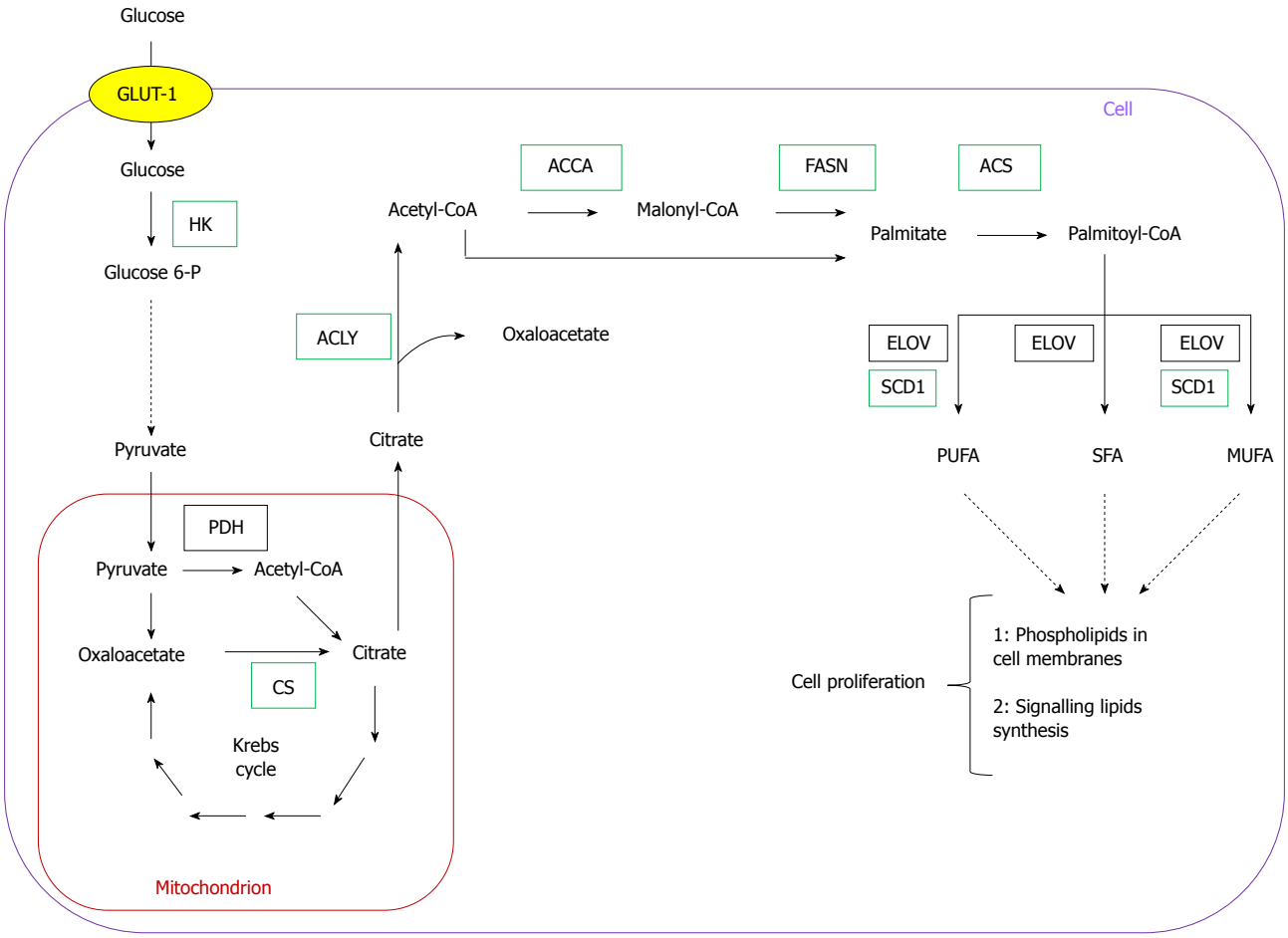

Figure 5 Fatty acid synthesis in pancreatic cancer. The up-regulated enzymes are marked in green. GLUT-1: Glucose transporter 1; HK: Hexokinase; glucose 6-P: Glucose 6-phosphate; PDH: Pyruvate dehydrogenase; CS: Citrate synthase; ACLY: ATP citrate lyase; ACCA: Acetyl-CoA carboxylase; FASN: Fatty acid synthase; ACS: Acyl-CoA synthetase, ELOV: Elongase; SCD: Stearoyl-CoA desaturase; PUFA: Polyunsaturated fatty acids; MUFA: Monounsaturated fatty acids; SFA: Saturated fatty acids.

enzyme of oxidative phase, controlling NADPH production) is increased putatively, due to p53 deficiency[19]. p53 inhibits G6PDH through direct binding, and its loss leads to the up-regulation of the oxidative PPP phase in cancer cells[73,124]. Taken together, these data suggest that similar to other malignancies, the increased glucose flux (both by glycolytic and pentose phosphate pathway) is integrated with the enhanced biosynthesis of lipids in PC cells. Pathways involved in the conversion of glucose to lipids in PC cells are presented in Figure 5.

The lipids formed in cancer cells play two important roles. Firstly, they are building blocks for cell membrane formation during cell proliferation (mainly cholesterol, phosphatidylcholine, phsphatidylserine, phosphatidylethanolamine). Secondly, they play an important role as signaling molecules (phosphatidylinositol, phosphatidic acid, diacylglycerol), or substrates for posttranslational protein modification, including palmitoylation and prenylation[125]. Mammalian cancer cells rely mostly on saturated (SFAs) or monounsaturated FAs (MUFAs). MUFAs are less susceptible to peroxidation, thus increasing the resistance of cancer cells to oxidative stress[126]. Elevated level of MUFAs is maintained mostly by stearoyl-CoA desaturase 1 (SCD1). Inhibition of SCD1 activity in some tumors (*e.g.*, in prostate cancer) leads to inhibition of cancer cell growth. Diminished SCD1 activity is reflected by lower synthesis of phosphatidylinositol, which participates in AKT activation, crucial for cancer development and growth. Additionally inhibition of SCD1 blocks oncogenic transformation of KRAS necessary for activation of this gene and further tumor growth[127]. As SCD1 is very active in PDAC cells[128], and KRAS and AKT signaling pathway are important for their development and growth, SCD1 supposedly plays an essential role in pathogenesis of that malignancy via the same mechanism as in case of other tumors.

In the context of lipid synthesis, especially FASN activity, special attention should be paid to lipid rafts. Lipid rafts are cholesterol- and sphingolipid-rich membranous lipid domains, which contain several signaling and transport proteins. According to some authors, lipid rafts play an important role in health and disease, including carcinogenesis[129]. Lipid rafts rich in proteins of the caveolin family are referred to as caveolae. Caveolin-1 encoding gene expression is altered in some cancers including colon cancer[130,131], breast cancer[132], urothelial carcinoma[133], esophageal squamous cell carcinoma[134], and prostate cancer[135]. The overexpression of caveolin -1 in

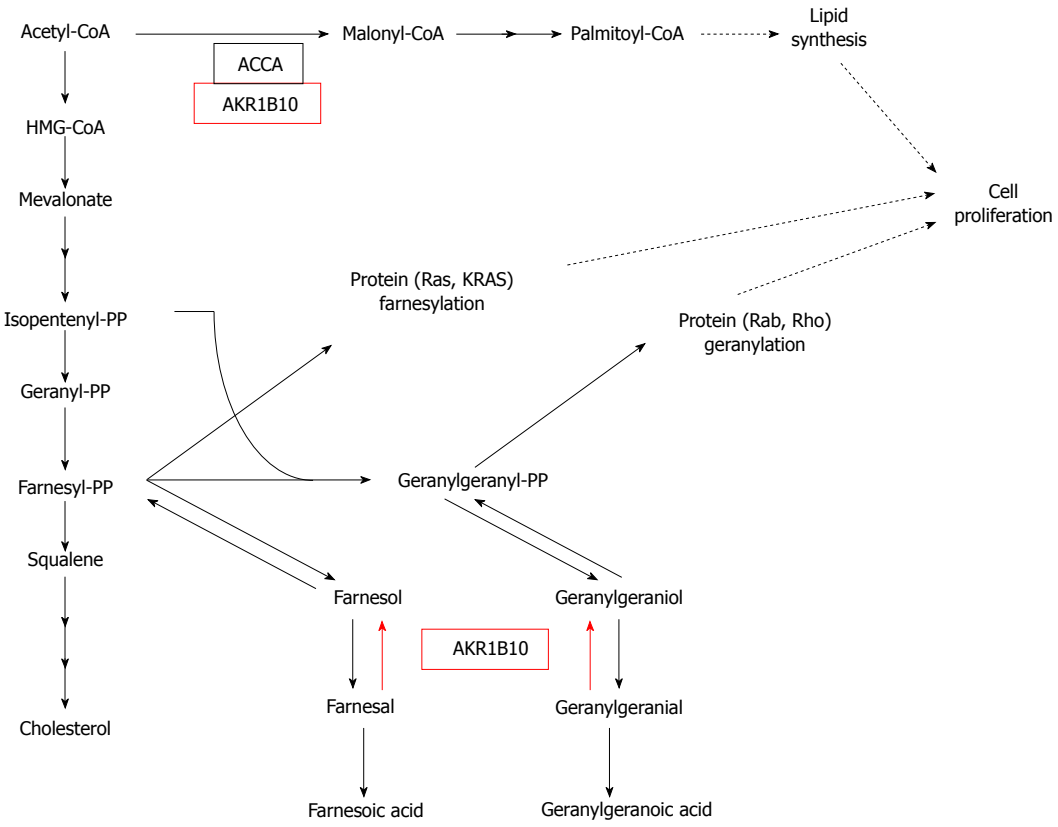

Figure 6 Possible role of aldo-keto reductase 1B10 in regulation of cell proliferation in pancreatic cancer. Binding of AKR1B10 results in stabilization of ACCA, and up-regulation of fatty acid synthesis. AKR1B10: Aldo-keto reductase family 1B10; ACCA: Acetyl-CoA carboxylase; Isopentenyl-PP: Isopentenyl diphosphate; Geranyl-PP: Geranyl diphosphate; Farnesyl-PP: Farnesyl diphosphate; Geranylgeranyl-PP: Geranylgeranyl diphosphate.

colon cancer cells is associated with elevated saturated to unsaturated FA ratio in cellular membrane[136]. Deregulation of caveolin-1 is also observed in PC cells[137]. Moreover, caveolin-1 and *FASN* are co-expressed in the cells of this malignancy. This phenomenon is consistent with histological grade and stage of the tumor (high expression of ceveolin-1 and *FASN* genes correspond with poor differentiation status)[104]. Thus, FASN and caveolin-1 were suggested as potential diagnostic and prognostic markers of PC and possible therapeutic targets[104].

Recently published data suggest that cancer cells do not rely solely on the *de novo* lipogenesis, but also utilize food-derived FAs for synthesis of phospholipids required for cell proliferation and lipid signaling[138,139]. This corroborates well with the evidence that a high dietary intake of fat constitutes potential risk factor of some malignancies[140]. Moreover, there is growing evidence that obesity, associated with elevated blood concentrations of FAs, modulates the risk and prognosis of certain cancers[141]. These findings suggest that, apart from lipogenesis, cancer cells can utilize FAs present in blood (derived from VLDL and chylomicrons or from adipose tissue) for their growth. This fact may partly explain why many promising lipogenic enzymes inhibitors tested succesfully in preclinical studies did not confirm their efficacy in further clinical trials. Furthermore, apart from inhibition of lipogenesis, also reduced dietary lipid digestion and absorption, and decreased lipoprotein lipase and FAs uptake seem necessary for the control of cancer growth[139].

Recently, overexpression and oncogenic function of aldo-keto reductase family 1B10 (AKR1B10; A-aldo, K-keto, R-reductase), tightly associated with lipid metabolism in human PC cell lines, has been reported[142]. AKR protein family consists of enzymes which catalyze the reaction: alcohol + $NADP^+$ → aldehyde (or ketone) + NADPH + H^+. These enzymes are expressed in numerous human organs/tissues. AKR1B10, the enzyme specific to such substrates as farnesal, geranylgeranial, retinal, and carbonyls[143-146], is overexpressed in certain malignancies, especially in tobacco-related cancers, including non-small cell lung carcinoma[147] and PC[142]. Oncogenic function of AKR1B10 is associated with protein farnesylation and up-regulation of FA synthesis by stabilization of ACCA[142,148]. Farnesyl diphosphate is a precursor of cholesterol biosynthesis and a substrate for protein farnesylation, which plays an important role in carcinogenesis[149]. Conversion of farnesyl diphosphate to farnesol diminishes its intracellular level, and, consequently, protein farnesylation. Farnesol can be further converted to farnesal, then oxidized to farnesoic acid. If the activity of AKR1B10 is high (as in the case of PC cells), farnesal is reduced to farnesol, following the reaction pattern: farnesal + NADPH + H^+ → farnesol + $NADP^+$. Farnesol can be re-phosphorylated to farnesyl diphosphate, increasing the ability for protein farnesylation (Figure 6). Farnesyl diphosphate, together with isopentenyl di-

phosphate, is converted to geranylgeranyl diphosphate, a substrate for protein geranylation. A geranylgeranyl diphosphate, *e.g.*, farnesyl diphosphate, can be converted to geranylgeranoic acid (*via* geranylgeraniol and geranylgeranial) (Figure 6). Similarly, high activity of AKR1B10 may cause the reversed conversion of geranylgeranial to geranylgeranyl diphospate, a substrate for protein geranylation (Figure 6). siRNA-mediated silencing of AKR1B10, knockdown of AKR1B10, or inhibition of the enzyme activity lead to decrease in protein prenylation[142]. Membrane-bound KRAS protein of PC cells, a product of point mutation in KRAS is activated by prenylation. If the expression of AKR1B10 is diminished, the activity of membrane-bound KRAS protein decreases in pancreatic cell lines[142]. Thus, the deactivation of AKR1B10 and resultant inhibition of the prenylation (fanezylation, geranylgeranylation) of protein (*e.g.*, KRAS), may constitute a promising target for PC treatment.

3-hydroxy-methylglutaryl-CoA reductase (HMG-CoA reductase) is a key enzyme of cholesterol synthesis pathway (Figure 6), which is inhibited by statins, prescribed to treat hypercholesterolemia. Since the reaction catalysed by HMG-CoA reductase provides substrate for cholesterol synthesis (that is of great importance in rapidly proliferating cancer cells), and also for isoprenoids necessary for prenylation of proteins, the application of statins as an antiproliferative drugs have been studied. Numerous *in vitro* studies, also with the use of PC cancer cells, provided promising results[150].

Cyclooxygenase-2 (COX-2) is another enzyme which plays an important role in lipid metabolism, namely in the conversion of arachidonic acid (released from membrane phospholipids by phospholipase A_2) to prostaglandins. COX-2 is overexpressed in many malignancies, including 45%-75% PCs[151-155]. This suggests, that this enzyme plays an important role in pancreatic carcinogenesis and chemoresistance of PC cells. Moreover, the overexpression of COX-2 in PC cells was postulated to be associated with greater invasiveness of this malignancy and promotion of angiogenesis. Recent data suggest that combination of COX-2 inhibitor (Celecoxib) with gemcitabine and irinotecan could be an active treatment for non-operable PC[152]. These clinical observations have been supported by the results of *in vitro* studies. Inhibition of COX-2 by non-steroidal anti-inflammatory drugs causes a dose-dependent block of pancreatic cell line proliferation[156]. According to recent reports, the anti-tumor activity of class I histone deacetylase (HDAC) inhibitors in human PC model is significantly improved by the simultaneous inhibition of COX-2[157]. Taken together, the results of clinical and *in vitro* observations suggest that COX-2 plays an important role in PC development. The up-regulation of COX-2 in PC cells and its role in carcinogenesis are probably related to inflammation. The anti-cancer action of COX-2 inhibitors is most likely associated with the reduction of inflammation that can contribute to cell proliferation. Several authors revealed that many malignancies, including PC, result from a chronic inflammatory process[158]. According to Jackson and Evers[151], several signaling pathways involving COX-2, NF-kappa B and phosphatidyl inositol 3-kinase may constitute a link between inflammation and carcinogenesis.

ABNORMAL LIPID METABOLISM AND CANCER PROGRESSION AND PROGNOSIS

Overexpression of *FASN* is associated with significantly enhanced proliferation of non-tumorigenic mammary[111] and prostate[159] epithelial cells. On the other hand, siRNA-mediated silencing of *FASN* gene expression or inhibition of FASN activity by pharmacological (synthetic or natural) agents leads to growth arrest of some cancer and normal cells[160-162]. Moreover, FASN inhibitors suppress the synthesis of DNA and induce apoptosis in cancer cell lines[163]. Previous studies confirmed the association between FASN activity and cell cycle progression[161,164]. However, activity of FASN was not reflected by cell cycle progression in some experimental models, *e.g.* MCF7 cell line[165]. Also siRNA-mediated knockdown of *FASN* gene expression did not cause a significant growth arrest in PC cell line (Panc-1)[105]. Therefore, the results published thus far do not present sufficient evidence for the role of FASN in cell cycle regulation, especially in PC cells. Nevertheless, the FASN knockdown in Panc-1 cells were revealed to show reduced resistance to gemcitabine[105].

The results of *in vitro* studies and clinical observations suggest that elevated expression of *FASN* gene in cancer cells is related to markedly worse prognosis. Overexpression of *FASN* gene was proved to be associated with cancer progression, higher risk of recurrence and shorter survival of patients with breast cancer[5,166], prostate cancer[167], melanoma[168], nephroblastoma[169], renal cell carcinoma[170], endometrial carcinoma[171], colorectal carcinoma[172], ovarian cancer[173], squamous cell carcinoma of the lung[174], head and neck squamous cell carcinoma[175], and squamous cell carcinoma of the tongue[176]. CD44 is a transmembrane glycoprotein which is involved in tumor progression and metastasis[177]. Interaction between CD44 and c-MET (tyrosine kinase), a proto-oncogene involved in several processes (including tumor growth, invasion, and metastasis)[178], is essential for activation of the latter and down-stream signaling in some malignancies[179]. Interestingly, inhibition of FASN and ACLY in human colorectal cancer cell lines (KM20, HCT116) is associated with reduced expression of CD44. This is attributed to attenuated activation of c-MET, AKT, FAK, and paxillin, factors affecting adhesion, migration and invasion of cancer cells[180]. The abovementioned phenomenon was reflected by lower metastatic potential of colorectal cancer cells. The data suggest a direct link between lipogenic enzyme activity (FASN and ACLY) and tumor progression to a metastatic phenotype. As the inhibition of FASN is related to decreased phosphorylation of c-Met in diffuse large B-cell lymphoma[181] and prostate cancer[182], one can surmise that lipogenesis is feature of

metastatic cancers, including PC. However, to date there are no evidence confirming this hypothesis.

Overexpression of *FASN* gene is associated with poor prognosis in PC patients[96,104,105]. As previously mentioned, the overexpression of *FASN* gene may be associated with gemcitabine resistance of PC cells[105], and the inhibition of FASN enhances the cytotoxicity of this agent[105]. Similar phenomenon was observed in the case of human breast cancer cells and ovarian cancer cells. According to Menendez group, the inhibition of FASN is associated with enhanced cytotoxicity of docetaxel, vinorelbine, paclitaxel, 5-fluorouracil, and herceptin in the Her-2 positive breast cancer cell lines and ovarian cancer cells[183-187].

SERUM FATTY ACID SYNTHASE LEVEL AND SERUM FATTY ACID PROFILE-POTENTIAL BIOMARKERS FOR PANCREATIC CANCER

At present there is no sufficiently specific and sensitive serum (plasma) marker of PC. Ca19-9, the most widely used marker of this malignancy (the sensitivity up to 80%), is also elevated in other conditions, including chronic pancreatitis and cholangitis, as well as in other tumors[188,189]. Moreover, Ca19-9 is not useful in detecting early stages of PC[190]. According to some authors, circulating micro-RNA (miR-21, mir-210, mir155, mir196a) could constitute novel diagnostic biomarkers of PC[191,192]. Proteomic analyses of human PCs revealed numerous differentially regulated proteins, which could be involved in the progression of this malignancy, and, consequently, could act as its biomarkers, determined in pancreatic juice and in serum[193]. Also up-regulation of numerous proteins, which can be used as biomarkers of PC, has been reported recently[194]. However, despite extensive studies, we still lack a valid approach for detection of PC, especially its early stages, and sufficiently specific and sensitive biomarkers of this malignancy.

The fact that cancer cells and the normal cells of surrounding tissues are characterized by differential expression patterns of *FASN* suggests that serum levels of FASN may constitute a good biomarker of malignancy. Indeed, up-regulation of *FASN* in cancer cells was proved to be associated with increased serum levels of this enzyme in patients with some malignancies. The serum FASN level measured by ELISA in breast, prostate, colon, and ovarian cancer patients was significantly higher than in healthy controls[195-197]. Moreover, an increase in the serum levels of FASN proved to be proportional to the clinical stage of colorectal cancer and breast cancer[196,198]. The ELISA-determined serum levels of FASN were also elevated in patients with PC and intraductal papillary mucinous neoplasm[98]. Interestingly, the serum FASN levels of most PC patients decreased after resection of this malignancy[98]. This suggests that the elevated serum level of FASN reflects its up-regulation in PC cells. However, increased levels of FASN were also found in sera of patients with chronic pancreatitis[98]. This suggests that this parameter is not a PC-specific biomarker. Nevertheless, the serum levels of FASN could potentially add to the panel of markers used in the monitoring of individuals at high risk of PC.

According to some authors, PC patients show increased proportion of total MUFA in all plasma lipid classes, a feature which is associated with increased delta 9 desaturase (SCD1) and delta 5 desaturase indices[128]. Moreover, the association between longer survival of PC patients and higher level of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and with lower SCD1 index was demonstrated[128]. Recently, Yabushita *et al*[199] documented a significant decrease in serum (and pancreatic) level of palmitoleic acid in an experimental model of PDAC, and suggested that this FA could serve as a biomarker of human PC. Palmitoleic acid is a monounsaturated FA (16:1 n-9). It can be synthesized from palmitic acid, the main product of FASN, or may originate from diet. Conversion of palmitic acid to palmitoleic acid is catalyzed by SCD1, which is up-regulated in some malignancies including PC[128,200]. The reason for decrease in palmitoleic acid in patients with PC is not clear, as due to higher activity of SCD1, elevated level of this FA should be rather anticipated. Despite unknown molecular basis for the decreased serum and tissue concentration of palmitoleic acid the diagnostic value of this finding should be verified in patients with PC. Chavarro *et al*[201] showed that blood levels of some MUFAs including myristoleic acid (14:1 n-5), palmitoleic acid, and oleic acid (18:1 n-9), were associated with higher incidence of prostate cancer. This relationship was the strongest in the case of palmitoleic acid.

Recently Zhang *et al*[202] reported that PC can be diagnosed by means of ^{1}H nuclear magnetic resonance (NMR)-based metabonomic profiles. These authors showed that numerous plasma metabolites, including lipids, are either elevated (*e.g.*, VLDL) or decreased (*e.g.*, HDL, LDL and 3-hydroxybutyrate) in patients with this malignancy.

Yabushita *et al*[199] revealed that serum chenodeoxycholic acid, a major constituent of bile acids (which play a key role in lipid digestion in the alimentary tract), is elevated in the experimental model of PDAC. Also Urayama *et al*[203] claimed on elevated serum levels of some bile acids (taurocholic acid and tauroursodeoxycholic acid) in PC patients.

Overall, several PC-characteristic features of lipids metabolism have been found: (1) elevated serum level of FASN; (2) elevated serum levels of EPA, DHA and VLDL; (3) decreased serum levels of palmitoleic acid, HDL, LDL and 3-hydroxybutyrate, and (4) elevated serum levels of bile acids. All these parameters could serve as additional markers of PC.

Up-regulation of lipogenic enzymes in PC cells and resultant enhanced synthesis of lipids[19,96,104,105] seem to occur early in tumorigenesis and can be associated with

Table 5 Lipogenic enzyme inhibitors that can be used as potential antitumor drugs

Enzyme name	Inhibitor	Type of neoplasm	Ref.
Fatty acid synthase (FASN)	Cerulenin	Breast cancer, ovarian cancer	[303] [304]
	C75	Breast cancer	[216]
		Pancreatic cancer	[232]
	Epigallocatechin-3-gallate (EGG)	Prostate cancer	[228]
	C93	Lung cancer	[305]
		Ovarian cancer	[306]
	Luteolin	Breast cancer, ovarian cancer	[228]
		Pancreatic cancer	[232]
	Orlistat	Prostate cancer	[307]
ATP citrate lyase (ACLY)	SB-204990	Lung cancer	[308]
	hydroxycitrate	Brest cancer	[18]
		Pancreatic cancer	[101]
Acetyl-CoA carboxylase (ACCA)	Soraphen A	Prostate cancer	[309]
	TOFA	Lung cancer, colon cancer	[310]
Stearoyl-CoA desaturase (SCD1)	CVT-11127	Lung cancer	[222]
	TOFA	Colon cancer	[225]
Acetyl-CoA synthetase (ACS)	Triacsin c	Various cancers cell lines	[311]

the progression of the disease. Therefore, metabolic imaging with lipid precursor tracers: ^{11}C-acetate, ^{18}F-fluoroacetate (as a substrates for FA synthesis), and ^{11}C-choline, ^{18}F-fluorocholine (as a substrate for phosphatidylcholine synthesis), may constitute a novel imaging technique for diagnosis of PC, even at the very early stages of this malignancy. It is of note that ^{11}C-acetate and ^{11}C-choline have been successfully used for detecting primary prostate cancer, as well as metastases and recurrence of this malignancy[204]. However, both ^{11}C-acetate and ^{11}C-choline cannot be used in case of small metastatic foci[205]. Moreover, the sensitivity of ^{11}C-acetate in the detection of prostate cancer is decreased in patients whose PSA level is lower than 3 ng/mL[204]. Finally it should be remembered that the incorporation of ^{11}C-acetate (or its analogue ^{18}F-fluoroacetate) to lipids is determined not only by FASN activity, but also by the activity of acetyl-CoA synthetase[206].

ABNORMAL LIPID METABOLISM AS A PROMISING TARGET OF PANCREATIC CANCER TREATMENT

Chemotherapy provides only modest improvement in pancreatic cancer patients. Effective molecular therapeutic strategy requires characteristic features of the disease to be identified. As previously mentioned the values of some parameters of lipids synthesis, namely the expression of *FASN* gene and resultant activity of FASN, are significantly higher in cancer cells than in adjacent normal cells. This suggests that inhibition of FASN could constitute a selective therapeutic approach in cancer patients. Possible application of FASN as a therapeutic target is sustained by the results of many studies which showed that pharmacological blockade of this enzyme exerted cytostatic and cytotoxic effects to several tumor cells[97,109,125,207-216]. Pharmacological blockade of other enzymes involved in lipogenic pathway such as ACLY[18,209,217,218], ACCA[219-221], SCD1[222-225], and acyl-CoA synthetase[209], could also be an effective strategy for cancer treatment. Table 5 lists lipogenic enzymes inhibitor which can be potential antitumor drugs.

Similar to other malignancies, the overexpression of *FASN* observed in PC cells is associated with poor prognosis[96,104]. This suggest that FASN is involved in PC cell survival and its inhibition could constitute an effective strategy for PC treatment. Irresponsiveness to chemotherapy and radiotherapy is an important feature of PC. According to Yang *et al*[105], overexpression of *FASN* can be associated with resistance to gemcitabine and radiotherapy in PC patients. The exact molecular mechanism by which FASN induce gemcitabine resistance of PC cells is unknown. As the elevated expression of this molecule was proved to protect breast cancer cells from drug-induced apoptosis[165], also the FASN-induced resistance of PC cells to gemcitabine can result from similar mechanism. C75 (trans-4-carboxy-5-octyl-3-methylenebutyrolactone), a synthetic analog of natural cerulenin (isolated from Cephalosporum caerulens), is an inhibitor of FASN most often used in experimental models. This antitumor activity of this agent was documented in the case of human breast cancer[109], prostate cancer[226], ovary cancer[227] and mesothelioma[215] cell lines. Also many green tea polyphenols (*e.g.*, EGCG-epigallocatechin gallate or ECG-epicatechin gallate) and plant-derived flavonoids (such as luteoin) showed inhibitory effect to FASN[208]. Green tea polyphenols down-regulate *FASN* gene expression and induce apoptosis in human prostate cancer[228-230]. Luteolin (natural flavonoid) inhibits FASN *in vitro* and induces cytotoxic effects in breast, prostate cancer and hepatocellular carcinoma cells[231]. Moreover, the consumption of flavonoid rich foods was revealed to decrease the incidence of some malignancies[105]. Harris *et al*[232] studied the effect of FASN inhibitors (C75 and some phytochemicals) on the *in vitro* proliferation of PC

cells (MIA PaCa-2). They found that C75 and luteolin decreased proliferation of these cells at a similar dose. Also other tested phytochemicals, quercetin (flavonoid) and resveratrol (stilbenoid), inhibited the proliferation albeit, at significantly higher concentrations. The same authors revealed that the inhibitory effect of luteolin against PC cells results from three mechanisms: decreased synthesis of FA, and nucleic acids and decreased energy production. In contrast quercetin and resveratrol (natural inhibitors of FASN), which showed weaker inhibitory potential affect mainly glycogen metabolism. Collectively, the results published by Harris *et al*[232] suggest that the blockade of FASN by some flavonoids could lead to inhibition of pancreatic cells proliferation, similarly as in other cancer cells.

The results of clinical observations suggests that the incidence of cancer in diabetic patients, treated with metformin (an oral hypoglycemic drug, N,N'-dimethyl biguanide) is lower than in individuals with diabetes who do not receive this drug[233-236]. The anticancer properties of metformin were also confirmed by *in vitro* studies[237,238]. Recently, Nair *et al*[239] reported that metformin inhibits PC cell proliferation and tumor growth via down-regulation of Sp transcription factors and Sp regulated genes. Noticeably, *FASN* is one of the Sp regulated genes[240]. Thus, one can assume that the metformin induced blockade of PC cell proliferation and tumor growth is at least partially associated with indirect inhibition of FASN activity and lipid synthesis.

The anticancer potential of statins, inhibitors of HMG-CoA reductase also have been studied *in vitro* with various cancers cells lines. The antitumor effects of lipophilic statins (*e.g.*, lovastatin, simvastatin) resulted mainly from suppression of proliferation and promotion of apoptosis[150]. The chemopreventive effects of statins have been also reported in PC cell lines[241-243], and in mouse model of PC[244]. Available data from analyses on large human populations show, that daily intake of statins, in doses for cardiovascular event prevention, is not associated with the risk of PC[245-247]. However some recent data suggests that in subgroup of male smokers statins use may reduce the odds of PC[248], and is associated with better survival in diabetic patients[249]. The combination of statins and a FASN inhibitors used in an anticancer therapy would be of particular interest, but until now there are no data published regarding such approach.

In summary, the results presented above suggest that inhibitors of FASN (and inhibitors of other lipogenic enzymes) constitute promising anticancer agents. However, most of the known FASN inhibitors which can be potentially used as anticancer drugs displayed some side effects[250]. Nevertheless, the evidence of PC cells proliferation blockade resulting from direct or indirect inhibition of FASN, and potential involvement of FASN in gemcitabine (chemotherapeutic) resistance, substantiate further research on the role of this molecule in the biology and therapy of pancreatic malignancies. Moreover, there is an urgent need for specific/selective, side effect free inhibitors of FASN, which can be used in treatment of PC.

CONCLUSION

Similar to other malignancies, the reprogramming of lipid metabolism in PDAC, is closely connected with tumor development, growth, and progression. Hypoxia, activity of oncogenic factors, or the loss of tumor suppressors lead to significant changes in lipid biosynthesis and metabolism. KRAS, together with MYC and HIF1α, either increase the use of glucose and glutamine as substrates for FA synthesis, or regulate the lipogenesis directly. SFA and MUFA, (produced by FASN and SCD1 or taken up from blood), enhance the tumor growth by up-regulation of some oncogenic factors. FA built into phospholipids (together with caveolin-1) participate in the remodeling of cancer cell membrane structure. Other products of altered lipid metabolism, such as isoprene derivatives (farnesyl diphosphate or geranylgeranyl diphosphate), influence the activity of some proteins involved in tumorigenesis (enzymes and regulatory proteins) through their prenylation. Up-regulation of prostaglandin biosynthesis (from arachidonic acids) by COX2 links inflammation to PC development.

FASN is the most extensively studied enzyme involved in the lipid metabolism of PDAC cells. Its high activity in PC cells is associated with poor prognosis and increased resistance to chemo- or radiotherapy. Elevated serum levels of FASN, EPA, DHA or VLDL, and decreased serum levels of palmitoleic acid, HDL, LDL, or 3-hydroxybutyrate could serve as additional markers of PDAC. As the lipogenic activity of PDAC cells is higher than in normal cells, pharmacological inhibition of FASN and other lipogenic enzymes seems a promising therapeutic target. C75, some flavonoids, and metformin are good candidates for anticancer agents, but further research is required prior to their implementation to PDAC treatment.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Anaesthetic perioperative management of patients with pancreatic cancer

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Abstract

Pancreatic cancer remains a significant and unresolved therapeutic challenge. Currently, the only curative treatment for pancreatic cancer is surgical resection. Pancreatic surgery represents a technically demanding major abdominal procedure that can occasionally lead to a number of pathophysiological alterations resulting in increased morbidity and mortality. Systemic, rather than surgical complications, cause the majority of deaths. Because patients are increasingly referred to surgery with at advanced ages and because pancreatic surgery is extremely complex, anaesthesiologists and surgeons play a crucial role in preoperative evaluations and diagnoses for surgical intervention. The anaesthetist plays a key role in perioperative management and can significantly influence patient outcome. To optimise overall care, patients should be appropriately referred to tertiary centres, where multidisciplinary teams (surgical, medical, radiation oncologists, gastroenterologists, interventional radiologists and anaesthetists) work together and where close cooperation between surgeons and anaesthesiologists promotes the safe performance of major gastrointestinal surgeries with acceptable morbidity and mortality rates. In this review, we sought to provide simple daily recommendations to the clinicians who manage pancreatic surgery patients to make their work easier and suggest a joint approach between surgeons and anaesthesiologists in daily decision making.

Key words: Pancreatic cancer; Pancreatic surgery; Perioperative anaesthesia management

Core tip: Currently, the only curative treatment for pancreatic cancer is surgical resection. However, this type of surgery is still burdened by considerable morbidity due to its complexity and to the type of referred patients (elderly and with many co-morbidities). We believe that anaesthetic management with proper surgical approaches can play a key role in the outcome of the patient. Simple perioperative precautions in anaesthetic management (patient risk assessment, fluids management, prevention of surgical site infection, thromboprophylaxis, intraoperative ventilation, and intensive postoperative management) can help to ensure that these surgical operations are performed with reasonable assurance.

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INTRODUCTION

Pancreatic cancer (PC) is the fourth leading cause of

Table 1 A schematic representation of the integrated management of perioperative patients undergoing surgery for pancreatic cancer

Preoperative	Intraoperative	Postoperative
Informed patient consent	Combined general and epidural analgesia	Early nasogastric tube, catheter and drain removal
Preoperative risk assessment	Prevention of surgical site infection: Antimicrobial prophylaxis Avoid hypothermia Glucose control	Early oral nutrition/glycaemic control/goal-directed fluid therapy Pain relief/non-opioid oral analgesia
Evaluation and optimisation of preoperative physical conditions and medications	Blood transfusion management	Intensive postoperative ambulation and prevention of venous thromboembolism
Nutritional status	Intraoperative fluid management	Intensive respiratory rehabilitation
Risk stratification, rationale for thromboprophylaxis, and recommendations	Optimisation of intraoperative ventilation Intraoperative thromboprophylaxis	Intensive postoperative management

Modified from Grade *et al*[10].

cancer-related death in the United States and the sixth in Europe, with the lowest survival rate for any solid cancer worldwide[1]. It is the most lethal type of digestive cancer and exhibits a five year survival rate of 5% with a range that is correlated with staging and location. The main reason for this extremely poor prognosis is that less than 15% of patients are diagnosed with resectable tumours[2]. Currently, the only curative treatment for PC is surgical resection, although even for resectable tumours, cure is still rare (5-year survival rate of approximately 15%-20%)[3].

Pancreatic surgery represents a challenging and technically demanding major abdominal procedure that occasionally results in a number of pathophysiological alterations during the early postoperative period that account for increased rates of morbidity and mortality.

Systemic, rather than surgical complications, cause the majority of PC-related deaths[4]. More than 80% of PCs are diagnosed in patients older than 65 years. Many PC patients are or have been heavy smokers[5,6], and nearly 80% of PC patients have either frank diabetes or impaired glucose tolerance[7]; venous thromboembolism remains a major complication of PC[8]. For these reasons, PC patients who undergo a major abdominal surgery are at increased anaesthesiological risk. In the light of these issues, it is important to refer these patients to centres with a high volume of operations where a multidisciplinary approach is applied to improve the overall outcome. Moreover, careful patient selection is fundamental.

In this setting, the anaesthesiologist plays a crucial role during preoperative evaluation, which together with a proper surgical approach and a concerted effort with medical physicians, radiation oncologists, gastroenterologists and interventional radiologists is crucial for a favourable perioperative outcome[9]. Patient outcome can be significantly influenced by anaesthesiological management (Table 1), starting with patient stratification and selection, continuing throughout the surgical operation and finishing with postoperative care [intensive care unit (ICU), recommendations for the ward][10].

PREOPERATIVE MANAGEMENT

Informed patient consent

Despite recent developments in operative technique and postoperative care, pancreatic surgery remains associated with high morbidity and mortality. Postoperative complications such as primarily pancreatic fistula, haemorrhage, abscess, and delayed gastric emptying still occur at a frequency of 30% to 60%, resulting in a mortality rate of 1% to 5%[11]. For this reason and due to the lethality of the pancreatic cancer despite surgical treatment, the patient should be informed about the therapeutic procedure and any potential complications or disabilities to facilitate a conscious involvement in the decision-making process.

In the case of patients of advanced age who require pancreatic surgery, formal mental status testing can help determine whether a patient can be considered capable of making this type of decision.

Dementia is an extreme predictor of poor outcome, exhibiting surgical mortality rates that are increased by 52%[12]. The decision to classify an elderly patient eligible for surgery cannot exclude preoperative mental status.

Preoperative risk assessment

A complete history, physical, laboratory examinations, and an assessment of the surgical risks should be included in the preoperative evaluation of an elective surgery.

Currently, the definition of preoperative risk remains vague and difficult to standardise, as it is influenced by many variables attributed to patient- and surgery-specific variability[13]. Recently, a variety of scoring systems has been developed, and the Physiologic and Operative Severity Score for the Enumeration of Mortality and morbidity (POSSUM) model by Copeland *et al*[14] was recognised as the most effective for general surgery[15]. This model, which uses scores relating to 12 physiological and 6 operative variables, was developed to postoperatively predict 30-d mortality and morbidity. The application of the predictive POSSUM and P-POSSUM (Portsmouth modification of POSSUM)[16] models to cases of pancreatic surgery has generated conflicting results. The implementation of this scoring system in the routine practice has proven to be difficult, and a recent review by Wang *et al*[17] has found POSSUM to overpredict postoperative mortality. Despite these limitations, there is still a role for POSSUM as a useful tool in pancreatic surgery. Individual POSSUM scores should not preclude pancreatic resec-

tion in clinical practice but might help surgeons modify expectations of postoperative outcomes[18].

Due to the limitations of the POSSUM model, more trials are needed to adequately evaluate this scoring system in predicting postoperative mortality for pancreatic surgery.

Evaluation and optimisation of preoperative physical conditions and medications

A growing number of old patients benefits from a surgical procedure[19]. Age is an independent risk factor of postoperative mortality and postoperative complications and can cause a gradual progressive loss in the biological reserves for maintaining physiological homeostasis under stress. In addition, an increasing number of patients present with one or more age-related chronic conditions, which further decrease their ability to respond to stress. Cardiac and pulmonary diseases are the most frequently observed co-morbidities that anaesthetists and surgeons must manage during this complex surgery.

A complete history of prior medical and surgical conditions and a full medication list are particularly important[20,21].

Cardiovascular risk evaluation: Cardiovascular complications are among the most common and significant postoperative problems in elderly patients. A practical guideline for perioperative cardiovascular evaluation for non-cardiac surgery has been proposed by the American College of Cardiology and American Heart Association Task Force[22]. Patients should be assessed using an approach that considers clinical predictors, the risk of the proposed operation and the functional capacity.

Ageing is accompanied by increased vascular and ventricular stiffness, diastolic dysfunction and an increased risk of heart failure[23]. Diastolic dysfunction even with a normal or supranormal ejection fraction might elicit a significant effect on the perioperative outcome and management of elderly patients[12]. Diastolic dysfunction might significantly affect perioperative haemodynamics, response to fluid shifts, anaesthetic drugs and other perioperative medications.

Patients with cardiovascular diseases are sensitive to haemodynamic instability and often require increased filling pressures to generate an adequate cardiac output. The anaesthetist must carefully manage fluids during the operation to avoid overload or rapid volume administration. Moreover, the anaesthetist must maintain a normal haemoglobin value (Nair *et al*[24] demonstrated that anaemia was strongly associated with diastolic dysfunction in patients with coronary artery disease) and, if possible, must choose volatile anaesthetics that appear to improve diastolic parameters (in contrast to propofol, which elicits the opposite effect) as measured by echocardiography[25]. Thoracic epidural analgesia should be strongly suggested, not only for pain management and for decreasing respiratory complications but also because its use appears to improve cardiac function by improving the diastolic characteristics of the left ventricle[26,27].

Prophylactic perioperative β-blockade: In general, cardiovascular medication should not be discontinued prior to surgery. In the perioperative setting, β-blockers are not contraindicated in patients with diastolic heart failure and should be continued in patients with systolic heart failure. However, caution is warranted with the acute administration of β-blockers in situations of decompensating systolic heart failure. Nonetheless, given the risk of acute withdrawal, β-blockade in patients with coronary artery diseases or coronary artery disease risk factors should not be discontinued preoperatively. Rather, perioperatively increasing the dosage of the patient's β-blockade regimen would most likely be beneficial[28-30].

If a patient who is scheduled for elective pancreatic surgery requires a new prescription, it should be started at least 1 mo prior to the procedure to allow for dose adjustment[31,32].

Pulmonary risk evaluation: Pulmonary complications such as pneumonia, failure to wean, and postextubation respiratory failure represent the second most frequent types of postoperative complication following wound infection, with an estimated incidence rate ranging from 2.0% to 5.6% following surgery[33,34]. Pulmonary disease increases the risk of postoperative complications, accounting for 40% of postoperative complications and 20% of deaths[35]. Age-related changes, such as increased closing volumes and decreased expiratory flow rates can predispose older patients to pulmonary complications.

Some postoperative pulmonary complication (PPC) predictors after pancreatic surgery are summarised in Table 2 (modified from Canet *et al*[36]).

Identifying the patients who are at high risk for PPCs, can help the anaesthetist to design individually tailored management approaches[37-39]. Pharmacologic measures for managing these complications are either unavailable or limited, and as a result, treatments must be based on physical therapy and respiratory support ventilation.

Finally, the ability to predict PPCs would enable clinicians to give patients more precise risk assessments, thereby facilitating their decision making.

Nutritional status and mechanical bowel preparation

The prevalence of malnutrition is high in patients who are submitted for surgery and ranges from 35% to almost 60%[40]. Malnutrition has been consistently associated with impaired immunity[41] and can lead to increased complications, such as pressure ulcers, delayed wound healing, increased risk of infections, impaired muscular and respiratory functions[42], as well as increased mortality and poor clinical outcomes.

Nutritional status should be determined because nutritional deficiencies are common in patients who have undergone pancreatic resection for malignant tumours. Because malnutrition is potentially reversible with appropriate nutritional support, the early identification of high-risk patients is crucial, and preoperative malnutrition screening is required to identify and to treat the malnutrition[43]. Recently, the routine screening of patients to iden-

Table 2 Perioperative clinical predictors of postoperative pulmonary complication in pancreatic oncological surgery

Patient-related factors	Surgery-related factors	Preoperative testing-related factors
Congestive heart failure	Abdominal surgery	Serum albumin concentration < 2.5 g/dL
ASA score > 2	Surgery duration > 3 h	Anaemia (Hb < 10 g/dL)
Age > 65 yr	General anaesthesia	Low SpO2
Chronic obstructive pulmonary disease	Transfusions	Chest X ray
Functional dependence	Prolonged hospitalisation	
Weight loss		
Impaired sensorium		
Cigarette smoking		
Respiratory infections within the past month		

Modified from Canet *et al*[36]. ASA: American Society of Anesthesiologists.

tify risk of malnutrition has been recommended by many national, international, and specialist organisations[44,45]. The malnutrition universal screening tool (MUST) for adults was recently validated by several studies, which have demonstrated that as a screening procedure, MUST is rapid and easy to use[46,47].

The MUST appears to be a valid and easy screening tool for pancreatic surgery[20], which can identify patients at high risk for major complications and death. Furthermore, the MUST can prompt the implementation of effective nutritional interventions to reduce poor outcomes and thereby optimise the use of postoperative critical care beds and hospital resources.

As soon as malnutrition is recognised, preoperative nutritional supplements should be provided when possible. This supplementation can include high-energy foods, vitamins, enteral feedings, or, if necessary, total parenteral nutrition.

Mechanical bowel preparation

"Enhanced recovery" or "fast-track" (FT) programmes, which were first developed by Kehlet[48], are structured interdisciplinary strategies that have been introduced to optimise peri-operative care and to accelerate post-operative recovery[49]. A major intervention principle of this approach is the avoidance of preoperative mechanical bowel preparation (MBP), which has been employed as a preventative measure in gastrointestinal surgery for more than a century as an essential factor for avoiding infectious complications and anastomotic dehiscence. FT programmes, which exclude MBP, have been proposed more often in other surgical fields (elective colorectal, gastro-oesophageal and aortic surgery) and rarely have been applied to liver and pancreatic surgery[50]. The application of MBP in this type of surgery has been evaluated by limited studies (a retrospective case-control study by the Jefferson University[51] and a review by Salvia *et al*[52]), which have shown that it did not improve perioperative outcomes. At our institution, MBP has been excluded from clinical practice in pancreatic surgery. A recent review examined and compared the application of FT protocols with standard care in elective liver and pancreatic surgeries, showing that FT programmes can enhance post-operative recovery and reduce the length of hospital stays with no increase in adverse events, such as re-admissions, morbidity or mortality[53,54]. The avoidance of MBP, together with other measures including the application of epidural analgesia, the prevention of intra-operative hypothermia, fluid restriction, post-operative nutritional care and early mobilisation, collectively represent essential elements of a FT programme that is warranted for complex surgical operations such as pancreatic resection[55,56]. In our experience FT programmes for hepatopancreatic resections appear to be safe and associated with a reduction in the length of hospital stays.

Risk stratification, rationale for thromboprophylaxis, and recommendations

In patients undergoing general and abdominal-pelvic surgery, the risk of venous thromboembolism (VTE) varies depending on both patient- and procedure-specific factors[57]. Pancreatic cancer is among the most common malignancies associated with thrombosis, as it occurs in 50% of total patients[58]. Prophylaxis against postoperative venous thromboembolism should be tailored to the patient's level of risk. A model (the Caprini score) that can potentially be used for such purposes estimates VTE risk by adding points for various VTE risk factors[59].

Pharmacological prophylaxis reduces the risk of pulmonary embolism by 75% in general surgical patients and by 57% in medical patients[60]. The use of low-molecular-weight heparins (LMWHs) to prevent thrombotic events in these patients is a common and well-documented practice.

Current recommendations strongly advise effective and preventive strategies for all hospitalised patients who are defined as moderate to high risk for VTE and are awaiting pancreatic surgery.

LMWHs appear to be effective and are potentially associated with a lower risk of bleeding when the first dose is administered 12 h preoperatively[57,61]. We recommend the administration of LMWH from the day prior to surgery to all patients scheduled for pancreatic cancer surgery.

In the case of patients who are receiving anticoagulants or antiplatelet therapy and require an elective surgery or procedure, the actual guidelines addressing their management are underlined in Table 3 and are modified from Douketis *et al*[62].

INTRAOPERATIVE MANAGEMENT

Combined general and epidural anaesthesia

The use of thoracic epidurals is widespread for intraoperative and postoperative analgesia. Thoracic epidural anaesthesia (TEA) reduces sympathetic activity, thereby influencing the perioperative function of vital organ systems. Thoracic epidural anaesthesia has been used widely to provide excellent pain relief, to attenuate the catabolic

Table 3 Guidelines on the prophylaxis of venous thromboembolism and antiplatelet and anticoagulant management adjusted according to recent guidelines

In patients receiving bridging anticoagulation with a therapeutic-dose Ⅳ of unfractionated heparin, treatment is recommended to be stopped no later than at 4 to 6 h prior to surgery
In patients receiving bridging anticoagulation with a therapeutic-dose of LMWH, the last preoperative dose of LMWH is recommended to be administered at approximately 24 h prior to surgery instead of at 12 h prior to surgery
In patients receiving bridging anticoagulation with a therapeutic-dose of LMWH and are undergoing high-bleeding-risk surgery, resumption of the therapeutic dose of LMWH is recommended at 48 to 72 h after surgery instead of within 24 h following surgery
In moderate-to-high-risk patients receiving acetylsalicylic acid who require non-cardiac surgery, treatment with acetylsalicylic acid is recommended to be continued around the time of surgery instead of discontinued at 7 to 10 d prior to surgery
In patients with a coronary stent who require surgery, deferment of surgery is recommended at 6 wk or 6 mo after the placement of a bare-metal or drug-eluting stent, respectively, instead of initiating surgery during these time periods
In patients requiring surgery within 6 wk or 6 mo of the placement of a bare-metal or drug-eluting stent, respectively, continuing perioperative antiplatelet therapy is recommended instead of stopping therapy at 7 to 10 d prior to surgery

Modified from Douketis *et al*[62]. LMWHs: Low-molecular-weight heparins.

response to abdominal surgery, to lower the incidence of pulmonary morbidity, to decrease the cardiac metabolic demand, to reduce the risk of thromboembolic complications, to promote the recovery of intestinal function and to minimise motor blockade[63,64]. Moreover, epidural anaesthesia and mild hypercapnia have been shown to increase subcutaneous tissue oxygenation[65].

The combination of general anaesthesia and thoracic epidural anaesthesia has become the technique of choice at many institutions for major abdominal surgery[66,67].

Recent studies have suggested that for some types of cancer, TEA might also reduce the rate of recurrence after surgical resection. The possibility of reducing tumour recurrence makes the combination of general anaesthesia and TEA even more appealing, despite the existence of certain contraindications[68,69].

TEA represents a powerful tool that is available to anaesthesiologists for perioperative intervention in pancreatic surgery. At our University Medical Centre, we strongly address its use in the context of multimodal intervention.

Prevention of surgical site infection

Surgical site infections continue to represent a substantial source of morbidity and mortality in the surgical patient population. They are the second most common cause of nosocomial infection after urinary tract infections and account for approximately 17% of all hospital-acquired infections[70].

Increasing evidence indicates that anaesthesiologists play a prominent role in the prevention of surgical site infections. Anaesthesiologists are involved in the administration of antibiotics, in the use of supplemental oxygen, in the maintenance of normothermia and normoglycaemia, in the perioperative fluid management and in the administration of blood transfusions[71,72]. Therefore, decreasing surgical site infections depends on the optimisation of some perioperative conditions, which are generally controlled by anaesthesiologists.

Antimicrobial prophylaxis

The anaesthesiologist can play a simple but effective role in the prevention of surgical site infections by ensuring the administration of appropriate antimicrobial prophylaxis[73,74].

Current recommendations state that the infusion of the first dose of drug should begin within 30-60 min of incision. This period can be lengthened to 120 min for drugs such as vancomycin, where high infusion rates have been associated with complications[75]. The drugs used should be defined in advance for each intervention, including alternatives in the event that the patient presents with any contraindication for the frontline antibiotics. The determination of the ideal preoperative antibiotic therapy for a patient who is awaiting pancreatic surgery requires efforts by a multidisciplinary team (anaesthesiologist, surgeon and microbiologist). A proper and effective antimicrobial prophylaxis should be based upon the application of a standard protocol and quality management[76].

Concerning the duration and dosage of prophylaxis, the guidelines generally recommended a single standard intravenous therapeutic dose of antibiotic in the majority of procedures. Repeated doses have only been indicated in special circumstances such as prolonged surgery with a duration longer than the half-life of the antibiotic used or cases of major blood loss. This recommendation is based on published evidence, which suggested that the administration of short-duration prophylaxis is equally effective as longer-duration prophylaxis in the prevention of surgical site infections[77,78]. It is advisable to administer at least two antibiotic doses during pancreatic surgery.

Avoid hypothermia

Mild perioperative hypothermia (core body temperature 34-36 ℃) is commonly observed in surgical patients. The complications of mild perioperative hypothermia have been studied extensively and include increased duration of hospitalisation, increased intraoperative blood loss and transfusion requirements, increased adverse cardiac events, and an increase in patient thermal discomfort in the recovery room[79,80]. The effects of mild hypothermia on surgical site infections have also been studied. The major relation between hypothermia and increased surgi-

cal site infections is thought to be a decrease in subcutaneous tissue perfusion mediated by vasoconstriction[81,82]. The reduced oxygenation of the wound is responsible for reduced oxidative killing elicited by neutrophils and for the reduced production of superoxide radicals for any given oxygen tension[80].

Intraoperative core temperature monitoring (oesophageal temperature probe) and adequate control of body temperature are essential during pancreatic cancer surgery[83]. Heat loss during the first hour of anaesthesia is generally a result of the redistribution of core-to-peripheral temperature gradients caused by an anaesthetic-induced decrease in vasoconstriction. The exposure of the large bowel, significant amounts of fluids administered, and long surgical procedures represent other causes of intraoperative hypothermia. Actively pre-warming patients for 2 h prior to the induction of either general or regional anaesthesia[80] using forced-air warming blankets together with fluid-warming systems represents an important way to keep patients normothermic[84].

Glucose control

Hyperglycaemia is associated with an increased risk of morbidity and mortality[85]. Several studies have shown the negative effects of hyperglycaemic phases during hospitalisation on the rate of nosocomial infections, length of hospital stay and mortality[71,86]. In a recent trial, the use of insulin infusions to maintain serum glucose at less than 110 mg/dL in critically ill patients decreased the mortality rate from 8.0% to 4.6%, regardless of diabetic status[87]. In subsequent studies, the concept of intensive glucose control was modified towards less-extreme blood glucose levels because of dangerous hypoglycaemic episodes that were attributable for worse patients outcomes than that originally reported[88,89]. Intraoperative glucose control should be a standard practice during long and complex surgical procedures to reduce perioperative complications.

The optimal glucose level during the perioperative period has not been prospectively investigated, and the available data from recent reports do not indicate a specific threshold for the treatment of hyperglycaemia. There is some evidence that keeping glucose levels within a range of 110-180 mg/dL and not limiting the treatment to values higher than 200 mg/dL is safe and appropriate.

It is important not only to limit glucose control during the intraoperative period but also to continue insulin infusion during the postoperative period. The frequent and precise measurement of glycaemia must become a standard of pancreatic cancer patient management both during surgical procedures as well as during the postoperative period[90].

Blood transfusion management

Several published studies have demonstrated how blood product transfusions increase the postoperative risk of infection[91,92].

Published guidelines generally concur that although transfusions are not beneficial when the haemoglobin concentrations are greater than 100 g/L, they confer benefit when the haemoglobin concentrations are less than 60-70 g/L. Studies that have described transfusion management in Jehovah's witnesses have shown that morbidity and mortality only increase postoperatively for each gram of decrement when the haemoglobin concentration is less than 70 g/L[93]. Patients with cardiovascular diseases exhibit a significantly increased rate of postoperative mortality, and for this reason, the transfusion trigger should be different for patients with or without cardiovascular disease[94,95]. Although multiple trials have assessed the effects of transfusion thresholds on patient outcome, the literature is insufficient for defining a transfusion trigger in surgical patients with substantial blood loss. In the light of recent findings, the transfusion management of surgical patients should be patient specific and should not be based on arbitrary laboratory values but guided by patient covariables[96-99]. As underlined by the recent guidelines on perioperative bleeding management of the European Society of Anaesthesiology, we suggest a target haemoglobin concentration of 7-9 g/dL and the guidance of transfusions based on levels of serum lactate, base deficit, and central venous oxygen saturation[100].

Intraoperative fluid management

Optimal perioperative fluid management remains highly challenging, particularly in patients undergoing major abdominal surgery[101-103]. Perioperative physicians generally administer intravenous fluids to replace fasting deficits, third space losses, and blood loss to maintain adequate cardiac output, blood pressure, and urine output.

Fluid excess can have a negative impact on cardiac, pulmonary, bowel function and wound healing, predisposing the patient to tissue oedema and anastomotic breakdown[104,105]. In contrast, excessive fluid restriction can expose the patient to hypovolaemia and hypoperfusion[106]. Surgery causes inflammation and a corresponding release of mediators that can induce local tissue oedema[107]. Anaesthetists generally manage perioperative fluid administration by using unmonitored fixed fluid regimens and estimating fluid loss.

In recent years, restrictive fluid management has replaced this approach, and the concept of fast-track surgery has challenged the traditional administration of large amounts of fluids during surgery[108,109].

These findings have prompted fervent discussion on how liberal or restrictive perioperative fluid management should be applied, and several randomised controlled trial have attempted to settle this issue[104,108,110,111].

Due to the lack of consensus on the optimal implementation of fluid management, a new and more precise approach based on goal-directed fluid therapy and individualised fluid administration has been developed[103]. Goal-directed fluid optimisation has markedly increased tissue oxygen tension and microcirculatory perfusion in both healthy and perianastomotic tissues compared to the restricted fluid strategy[106,112,113].

Central venous pressure (CVP) remains the most

widely used clinical marker of volume status, despite numerous studies indicating no association between CVP and circulating blood volume[114]. Because of this limitation, central venous and pulmonary artery occlusion pressures, which are the only variables for guided fluid therapy and optimised preload, are not recommended. Dynamic parameters such as stroke volume variation or pulse pressure variation provide a more favourable prediction of fluid responsiveness. Individualised goal-directed fluid therapy, particularly oesophageal Doppler-guided fluid optimisation, has been shown to improve patient outcomes and to reduce the length of hospital stays compared with conventional fluid replacement[115]. Doppler-guided fluid boluses appear to improve clinical outcomes, particularly in elderly and frail patients[116,117]. This method, however, cannot be universally performed for practical and financial reasons[118].

Using a "goal-directed" approach, it is generally possible to replace lost plasma, whereas the extracellular compartment cannot currently be monitored. Therefore, losses from the latter should be replaced based on the protocol suggested by Chappell *et al*[101], which involves the substitution of insensible perspiration with 1 mL/kg per hour during abdominal surgery and does not include the possibility of primary fluid consumption by the third space, the existence of which is denied[119].

The optimal solution for volume replacement and optimisation remains an ongoing issue of heated debate. The goal of perioperative fluid management is to maintain fluid balance and to minimise the possible risks by choosing the right fluid at the right time.

Colloids are criticised because of their ability to diffuse into the interstitium, making further extravasation more likely[120], because of the cumulative and persistent effects related to their infusion[121] and, finally, because of safety concerns. Recent studies of the potential increase in the risk of bleeding and acute kidney injury following the application of various colloids have shown that the use of hydroxyethyl starch appears to be associated with an increased need for dialysis[122] and might even increase mortality in patients with sepsis[123].

Current evidence suggests that beyond fluid composition, the timing and volume of the administered fluid represent two additional factors that are likely to influence perioperative patient outcome. For patients with mild-to-moderate volume deficits, crystalloids are still the first choice. In the case of severe volume depletion, we recommend starting fluid resuscitation with a colloid to rapidly reverse volume deficits and ensure oxygenation and then to switch to crystalloids once the patient approaches euvolaemia.

Goal-directed fluid management enables appropriate use of fluids, vasopressors, and inotropes, and results in improved outcomes. The vasodilatatory effect of anaesthetic cannot be ignored and must be expected to terminate at the end of surgery. Treating vasodilatation with crystalloids or colloids can be a mistake in all euvolaemic patient, whereas vasopressor infusion during surgical operation can help in avoiding excessive fluid overload[124,125].

Optimisation of intraoperative ventilation

Postoperative pulmonary complications following major upper abdominal surgery increase morbidity, mortality, the length of hospital stay and costs[33]. Reduced lung inflation represents one of the basic mechanisms of postoperative pulmonary complications. The adjustment of the body positioning from upright to supine itself can reduce the resting lung volume by approximately 0.8-1.0[126]. The additive effect of supine positioning, general anaesthesia, and abdominal incisions significantly reduces functional residual capacity and increases airway resistance. In addition, during the induction of anaesthesia, most of the general anaesthetics further reduce functional residual capacity. The combination of these effects predisposes patients to atelectasis with the risks of hypoxemia and infection. Additionally, postoperative pain and the use of analgesics can contribute to a reduced tidal volume and impaired clearing of secretions, depending on adequate coughing and deep breathing[126,127].

Mechanical ventilation is mandatory in patients undergoing general anaesthesia. High tidal volumes can overdistend non-injured lungs, particularly in non-dependent lung tissues. The non-aerated atelectatic lung regions are prone to repeated collapse and re-expansion of the alveoli, causing shear stress and diffuse mechanical damage of the alveoli. During surgical procedures, both phenomena can induce stress in non-injured lung tissues, triggering local inflammation[128,129]. Retrospective and prospective studies have shown the potential beneficial effects of reduced tidal volumes in patients who are on short-term mechanical ventilation following surgery[130]. Protective mechanical ventilation using reduced tidal volumes can accordingly reduce ventilator-associated lung injury. The application of positive end expiratory pressure (PEEP) can prevent alveolar collapse and atelectasis formation, and recruitment manoeuvres can support the beneficial effects of PEEP during short-term ventilation[131]. Effective anaesthesiological management during pancreatic surgery should involve the application of a protective ventilation strategy (lower tidal volumes < 8 mL/kg, PEEP = 6-12 mmHg and recruitment manoeuvres) to improve respiratory function during the postoperative period following abdominal surgery and to reduce the clinical signs of pulmonary infection during the postoperative period[132].

Intraoperative thromboprophylaxis

The use of LMWHs to prevent thrombotic events in these patients represents a common and well-documented practice. Effective pharmacological thromboprophylaxis includes the administration of LMWH from the day prior to the surgery. In addition to this useful approach mechanical prophylaxis including graduated compression stockings and intermittent pneumatic compression is highly recommended during the surgical operation and during the postoperative period until the risk of bleeding has diminished and the application of new pharmacological prophylaxis might be initiated[57,60].

Thromboelastography can play a potential role, despite its limitations, as a valuable tool for the evaluation of the entire perioperative coagulation process and hypercoagulability changes, as well as for increasing patient safety through more effective management of antithrombotic therapy[133,134].

POSTOPERATIVE MANAGEMENT

Over the past 20 years, surgery and anaesthesia for patients undergoing abdominal surgery have undergone immense development. A novel concept of perioperative patient care following surgical abdominal procedures has emerged. Fast track programmes, a new concept of enhanced recovery after surgery and the implementation of multimodal rehabilitation, have heavily influenced this modern change, optimising perioperative care, accelerating recovery and reducing hospital stays and costs. The objective of this integrated approach between surgeon, anaesthetist, nurses and physiotherapist is to reduce the impact of surgery on patient homeostasis. The main pillars of this new management are those shared by fast track surgery and can be summarised as follows: (1) reduction of surgical invasiveness (early removal of drains, nasogastric tube, small incisions, pharmacological stimulation of the gut); (2) pain relief/non-opioid oral analgesia; (3) early oral nutrition/goal-directed fluid therapy; (4) intensive postoperative ambulation and prevention of venous thromboembolism; and (5) intensive respiratory rehabilitation.

All of these basic points, combined with the prevention of intraoperative hypothermia, neural blockades[135], maintenance of euglycaemia, and the development of goal-directed fluid therapy contribute to the reduction of surgical stress.

A systematic review of the literature regarding perioperative care in pancreatic cancer surgery has revealed a limited number of studies providing low levels of evidence[50,54,136]. Despite their potential weaknesses, the studies detailed above have demonstrated that implementation of fast-track peri-operative care pathways is feasible in pancreatic surgery and can be associated with reduced length of stay, reduced relevant hospital costs and no increase in morbidity, 30-d mortality or re-admission rates.

Early nasogastric tube, catheter and drain removal

Nasogastric tube: Nasogastric tubes have been routinely used following abdominal surgery until normal bowel function is restored, following the notion that gastric decompression resulting from decreased air and fluid accumulation can prevent abdominal distension, nausea and vomiting. Many studies have subsequently questioned this practice, advising against its routine use. In fact, prophylactic nasogastric tube aspiration is associated with pulmonary complications[137] and significant patient discomfort. A recent study on the implementation of fast-track recovery pathways in pancreatic surgery[138] has underlined the advantages of the early removal of nasogastric tubes and early oral feeding in terms of incidence of delayed gastric emptying and earlier bowel activity. Given the risk of pulmonary complications, significant patient discomfort and lack of benefit associated with prophylactic nasogastric tube aspiration, this practice should not be routinely used[139,140].

Consistent with a recent study, in our daily practice, we remove nasogastric tubes on postoperative day 1 only if the tube drainage amount is less than 300 mL or at the end of surgery in cases of distal pancreatectomy which makes delayed gastric emptying less frequent[52].

Abdominal drains: The presence of an abdominal drain represents a significant impediment to achieving early and appropriate levels of mobilisation. Several randomised trials have not found any benefit of prophylactic drains after surgical operations, such as cholecystectomy[141], colorectal surgery[142] or hepatectomy[143]. Rather, these prospective randomised studies found that routine drainage resulted in an increased frequency of complications and no difference in outcome.

Because pancreatic surgery is associated with high rates of morbidity, the purpose of prophylactic drainage is to prevent fluid collection and to aid in the early detection of anastomotic leak and associated haemorrhage. Following pancreatectomy, the use of a prophylactic drain is supported by the belief that the early detection of pancreatic fistulae through the measurement of amylase in the draining fluid will allow for the efficient management and the avoidance of major complications[144]. Despite reports of randomised, control trials and cohort studies that do not support the use of drains, most surgeons routinely place prophylactic intraperitoneal drains at the time of pancreatic resections[145,146]. Evidence-based practice guidelines for drain management during pancreatectomy remain to be established despite the remarkable number of studies that are available to help guide practice.

At our University Hospital, abandoning drainage during pancreatic surgery is believed to be unsafe, and according to Kaminsky *et al*[146], it is reasonable to suggest a practice of selective drainage based on the presence of risk factors. The presence of soft pancreas texture, a small pancreatic duct diameter, increased intraoperative blood loss (> 200 mL) and prolonged operative time are risk factors that reflect abdominal drains. In the case that patient is doing well and the drain amylase levels are below 5000 U/L, drains [on postoperative day 1 (POD 1)] can be safely removed on POD 3 in patients with low risk of pancreatic fistulae.

Early oral nutrition

The restoration of normal gastrointestinal function to allow adequate food intake and rapid recovery is one of the primary objectives of postoperative care. A meta-analysis of controlled trials of early enteral or oral versus 'nil by mouth' feeding after gastrointestinal surgery indicated no clear advantage to continued patient fasting after the elective gastrointestinal resection[147].

Concerning nutrition, studies have clearly found that

allowing eating/drinking until late the day prior to surgery and commencement of eating/drinking soon after surgery has many advantages[148,149]. Through the earlier intake of fluids and solids, the gastrointestinal system is less affected with an earlier initiation of normal intestinal activity.

An interesting review analysing which feeding routine was more favourable following pancreatoduodenectomy revealed no consensus in terms of postoperative nutrition of patients who had undergone pancreatic surgery. Current European guidelines recommend routine enteral feeding after pancreatoduodenectomy, whereas the American guidelines do not. Gerritsen *et al*[150] concluded that there is no evidence to support routine enteral or parenteral feeding after pancreatoduodenectomy, whereas the oral diet appears to be the best feeding strategy.

At our University Hospital, it is common to allow the patient to take clear liquids from POD 1 but not before 6 h postoperatively and a light diet from POD 2, in the absence of any complications. In patients at risk of postoperative complications such as pancreatic fistulae or abdominal collections, we advocate the use of combined parenteral and enteral nutrition[52].

Total pancreatectomy and postoperative glycaemic control

Total pancreatectomy, usually performed for the treatment of multifocal disease or in case of atrophic, soft, friable remnant pancreatic tissue is responsible of endocrine and exocrine insufficiency. In addition to the absence of insulin, the endocrine abnormalities accompanying total pancreatectomy include both glucagon and pancreatic polypeptide deficiencies, which appears to play a key role in the increased hepatic insulin resistance observed in pancreatogenic diabetes[151]. Moreover, following pancreatectomy, insulin receptors are upregulated peripherally, rendering patients uniquely sensitive to hormone replacement[152].

This type of diabetic condition is defined "pancreatogenetic" diabetes and is often considered to be different from type 1 and 2 diabetes. This diabetic state is commonly described as "brittle", as a result of enhanced peripheral insulin sensitivity, decreased hepatic insulin sensitivity and reduction of glucagon secretion. The resulting labile glycaemic control is characterized by periodic episodes of both hyper and hypoglycaemia[153,154].

In recent years, studies have shown that diabetes following total pancreatectomy is not necessarily associated with poor glycaemic control, and the majority of cases exhibit equivalent biochemical controls compared to the normal type 1 diabetic population[155,156].

Recently, the development of accurate, continuous blood glucose monitoring devices, particularly closed-loop systems, for computer-assisted blood glucose control in the intensive care unit have been reported to assist in obtaining favourable glycaemic control in patients with pancreatogenic diabetes following pancreatic resection[157].

The hyperglycaemia induced by surgical stress cannot be controlled using the conventional sliding scale method[158], whereas the perioperative use of an artificial endocrine pancreas enables strict glycaemic control of euglycaemia without severe hypoglycaemia[159,160].

Modern pancreatic enzyme formulations have improved exocrine insufficiency, facilitating glycaemic control due to the avoidance of malabsorption[155].

The enhanced patient understanding of the consequences of total pancreatectomy, early education on diabetes (all patients should consult an endocrinologist immediately following their operation), advances in medical therapies, and blood glucose monitoring might all have contributed to enhanced glycaemic control[161].

Goal-directed fluid therapy

Early oral nutrition has to be associated to the individualised postoperative fluid therapy that is administered in accordance to the optimisation of stroke volume. Dynamic parameters such as stroke volume or pulse pressure variation can provide a more favourable prediction of fluid responsiveness. Oesophageal Doppler-guided fluid optimisation has been shown to improve patient outcomes, although this method cannot be performed on conscious patients[116,117]. Fluid challenges and the leg-raising test can represent simple and valid alternatives[118]. Thus, oral nutrition has clearly be associated with a progressive decrease of intravenous fluids.

Pain relief/non opioid oral analgesia

One aim of fast track surgery is to obtain favourable pain control, which is intended to enable patient mobilisation, coughing and early nutrition. One of the modern principles for analgesia is the concept of opioid-sparing, which enhances recovery by avoiding the opioid-related side effects. In major abdominal procedures, the administration of continuous thoracic epidural analgesia with local anaesthetics has been demonstrated to be the most efficient technique to obtain optimal analgesia, allowing for early mobilisation, reducing postoperative ileus and pulmonary morbidity[162], and therefore acting as an important component of multimodal recovery strategies[163,164]. A mid-thoracic epidural activated prior to the initiation of surgery also blocks stress hormone release[165] and attenuates postoperative insulin resistance[166,167].

Fast-track clinical pathways in the peri-operative care of patients undergoing pancreatic resection provide for a catheter placed in the midthoracic level at T8/9 to achieve both analgesic and sympathetic blocks[168].

Small doses of epidural opioids have been shown to act in synergy with epidural local anaesthetics in providing analgesia, allowing reduced dosages of both agents[169].

For break-through pain, non-steroidal anti-inflammatory drugs and bolus epidural bupivacaine should be administered whilst the epidural is running. Non-steroidal anti-inflammatory drugs should be administered just prior to the removal of the epidural and continued until and/or after discharge.

As the optimal duration of continuous postoperative mid-thoracic epidural analgesia has not been established

in well-designed randomised trials, we suggest that two-to-three days might be a sufficient period for pancreatic surgery.

Patient-controlled analgesia using intravenous opioids does not provide the same efficient analgesia and elicits less beneficial physiological effects on surgical stress responses compared to local epidural anaesthetic techniques. However, it is performed whenever contraindications prevent the execution of peridural analgesia.

Intensive postoperative ambulation and prevention of venous thromboembolism

Among the standardised clinical pathways, which represent the basis of the fast-track programme, early mobilisation is a cornerstone. It has been shown to play a major role in postoperative functional recovery. Improved early ambulation can elicit beneficial effects in the resolution of postoperative ileus and can reduce the risk of lower extremity deep venous thrombosis. Furthermore, mobilisation might reduce pulmonary complications[170]. The risk for VTE, which is particularly high in this patient population, must be managed from the beginning of the preoperative period and continue during the entire surgical operation until the postoperative period as a result of early mobilisation and proper pharmacological thromboprophylaxis. At our University Hospital, we generally mobilise patients out of their beds for more than one hour from POD 1 and progressively increase the hours of mobilisation from POD 2. Patients who had undergone major abdominal surgery for gastrointestinal malignancies should be considered for post-discharge VTE prophylaxis for up to 4 wk following surgery during the following situations: residual or metastatic disease, obesity or previous history of VTE.

Intensive respiratory rehabilitation

Pulmonary complications following pancreatic resection occur in approximately one quarter of all patients[171]. Many pathophysiological modifications that occur under anaesthesia and/or following surgery can interact with each other, resulting in respiratory complications.

Reduced lung inflation is one of the basic causes of postoperative pulmonary dysfunction[172].

After upper abdominal and thoracic surgery, postoperative diaphragmatic dysfunction[173], which is the most important determinant of respiratory complications and atelectasis, is commonly observed and is caused by the mechanical compression of alveoli and the resorption of alveolar gases, which are the factors most commonly implicated in respiratory complications[174].

In recent years, breathing (deep breathing and directed cough) and chest wall physiotherapy have been introduced into clinical practice to prevent pulmonary complications. Physiotherapy includes a variety of manual treatments (postural drainage, percussion, clapping, vibration, or shaking) as well as the use of mechanical breathing devices (incentive spirometry, blow bottles, intermittent positive pressure breathing, and continuous positive airway pressure).

A systematic review showed that postoperative non-invasive ventilation, specifically continuous positive airway pressure (CPAP), improves hypoxaemia and reduces both postoperative complications and the requirement for intubation in patients undergoing abdominal surgery[170]. Furthermore, there is no specific study focusing on the role of chest physiotherapy after pancreatic resection; it is nonetheless included in the care plan at our institution. Every patient who has undergone pancreatic surgery is instructed to use a blow bottle (5 min/h) and undergoes an individualised exercise schedule that is designed by physiotherapists. Further, certain short courses of non-invasive mechanical ventilation (CPAP) can be performed as needed.

Intensive postoperative management

Despite continuous improvements in operative technique and perioperative management, the increasing age of patients undergoing major abdominal surgery exposes patients to an increasing number of postoperative complications, leading to increased morbidity, mortality, length of hospital stay, and hospital costs. Although the concept of fast-track surgery has questioned the traditional use of intensive care units, there is increasing evidence indicating that access to ICUs results in a more favourable impact on the outcomes of major abdominal surgeries.

In the case of pancreaticoduodenectomy, even high-volume centres report a major postoperative complication rate of approximately 20%[175]. Because of these observations, patients who undergo pancreatic cancer surgeries might benefit from admission to the ICU.

An ideal ICU model should involve the cooperation of the intensivists who primarily care for the patients with the primary physician and surgeon[176].

Current general concepts of fast track surgery have been implemented in intensive care units. Early mobilisation, early enteral feeding, and restrictive perioperative fluid management are generally performed at the ICUs of our institution. In addition to these programmes, ICU stays can offer extended haemodynamic monitoring, which is useful in goal-directed fluid therapy, the possibility of invasive and non-invasive ventilation, the continuous application of intravenous drugs or subsequently required extracorporeal procedures.

In summary, most patients who undergo elective pancreatic surgery for cancer do not necessary require intensive care admission, whereas high-risk patients might benefit from postoperative care in the ICUs. We suggest that surgical intensive care units play a pivotal role in the perioperative care of patients undergoing major abdominal surgeries, and patients with co-morbidities or elderly patients should be scheduled for intensive care treatment[177,178].

CONCLUSION

In recent decades, diagnostic modalities and the surgical treatments of PC have significantly progressed, de-

spite the fact that overall prognosis has only marginally changed. The management of patients affected by PC is complex and requires expertise in many fields. Multidisciplinary teams are necessary to optimise and improve the overall care and outcomes of patients. Because more patients are referred to surgery at an advanced age, a coordinated effort between surgeon and anaesthetist in terms of risk assessment is necessary, particularly for borderline resectable or unresectable disease cases (to spare the risk and cost of surgery for patients who are affected by advance disease and whose life expectancy might be potentially shortened by an unuseful and dangerous surgical operation)[179]. More favourable outcomes are attained if PC patients are appropriately referred to tertiary centres for assessment by surgical, medical and radiation oncologists, gastroenterologists, anaesthetists and other dedicated health care providers. The anaesthetist plays a key role in the preoperative assessment, intraoperative management and during the postoperative period assessment. For this reason, close cooperation between surgeons and anaesthesiologists is crucial for ensuring the safe performance of major gastrointestinal surgery with acceptable morbidity and mortality rates.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Involvement of substance P and the NK-1 receptor in pancreatic cancer

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Abstract

Pancreatic cancer is the fourth leading cause of cancer related-death for both men and women and the 1- and 5-year relative survival rates are 25% and 6%, respectively. Thus, it is urgent to investigate new antitumor drugs to improve the survival of pancreatic cancer patients. The peptide substance P (SP) has a widespread distribution throughout the body. After binding to the neurokinin-1 (NK-1) receptor, SP regulates biological functions related to cancer, such as tumor cell proliferation, neoangiogenesis, the migration of tumor cells for invasion, infiltration and metastasis, and it exerts an antiapoptotic effects on tumor cells. It is known that the SP/NK-1 receptor system is involved in pancreatic cancer progression: (1) pancreatic cancer cells and samples express NK-1 receptors; (2) the NK-1 receptor is overexpressed in pancreatic cancer cells in comparison with non-tumor cells; (3) nanomolar concentrations of SP induce pancreatic cancer cell proliferation; (4) NK-1 receptor antagonists inhibit pancreatic cell proliferation in a concentration-dependent manner, at a certain concentration, these antagonists inhibit 100% of tumor cells; (5) this antitumor action is mediated through the NK-1 receptor, and tumor cells die by apoptosis; and (6) NK-1 receptor antagonists inhibit angiogenesis in pancreatic cancer xenografts. All these data suggest that the SP/NK-1 receptor system could play an important role in the development of pancreatic cancer; that the NK-1 receptor could be a new promising therapeutic target in pancreatic cancer, and that NK-1 receptor antagonists could improve the treatment of pancreatic cancer.

Key words: Pancreas; Substance P; Neurokinin-1 receptor antagonists; Apoptosis; Antitumor; Angiogenesis; Metastasis; Pancreatic cancer

Core tip: The substance P (SP)/neurokinin-1 (NK-1) receptor system plays an important role in pancreatic cancer progression. Pancreatic cancer cells overexpress NK-1 receptors and SP promotes angiogenesis and the proliferation and the migration of pancreatic tumor cells. By contrast, NK-1 receptor antagonists, in a concentration-dependent manner, inhibit pancreatic cell proliferation (tumor cells die by apoptosis), have antiangiogenic properties in pancreatic cancer and block the migratory activity of pancreatic tumor cells. The antitumor action is mediated through the NK-1 receptor. Thus, the NK-1 receptor could be a new promising therapeutic target in pancreatic cancer and NK-1 receptor antagonists could improve pancreatic cancer treatment.

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INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer related-death for both men and women, with less than 5% survival at 5 years after diagnosis. In 2013, the American Cancer Society estimated 45220 new cases of pancreatic cancer in the United States and 38460 deaths from the disease. Treatment strategies have not succeeded in significantly extending patient survival, and neither have clinical outcomes improved substantially over the past 35 years; the overall 5-year survival rate remains dismal, at around 5%[1]. Pancreatic cancer remains a major unsolved health problem and conventional treatments having little impact on the course of the disease. Moreover, almost all patients with pancreatic cancer develop metastases, this being the primary reason for its lethality[2]. Accordingly, there is an urgent need to improve current therapies. Cytostatic drugs show a low safety profile and severe side effects, since they are not specific to tumor cells. Research should focus on drugs with the same or greater antitumor action but with fewer side effects. This can only be achieved if the drug is specific against pancreatic cancer cells and researchers are therefore seeking to identify novel molecular targets for blocking pancreatic cancer growth.

For some years, the expression and secretion of peptides by tumors has attracted increasing interest[3]. Substance P (SP) is an undecapeptide that is widely distributed throughout the body. It is derived from the preprotachykinin A gene and belongs to the tachykinin family of peptides. The biological actions of tachykinins (SP, neurokinin A, neurokinin B...) are mediated through the neurokinin-1 (NK-1), NK-2 and NK-3 receptors. SP has the highest affinity for the NK-1 receptor, which shows a widespread distribution throughout the body. This means that the biological actions (*e.g.*, pain, neurogenic inflammation, regulation of the cardiovascular system, mitogenesis...) exerted by the SP are mainly mediated by the NK-1 receptor[4,5]. Moreover, there are many data suggesting the involvement of the SP/NK-1 receptor system in cancer[5] (Figure 1 and Table 1). SP and NK-1 receptors have been detected in tumor cells and in intra- and peri-tumoral blood vessels[4-6]. SP induces mitogenesis in normal and tumor cells, protecting the latter from apoptosis, and controls the migration of tumor cells[4,7,8]. This is extremely important since the prevention of metastasis is a major goal in the treatment of tumors because over 90% of cancer deaths are derived not from the primary tumor but from the development of metastases. Moreover, it has recently been reported that the extravasation of tumor cells into the brain to form cerebral metastases may be an SP-mediated process[9]. More specifically, it has been reported that the SP/NK-1 receptor system is involved in pancreatic cancer by inducing pancreatic cancer proliferation, neoangiogenesis, and migration of pancreatic cancer cells (invasion, infiltration and metastasis). By contrast, NK-1 receptor antagonists inhibit pancreatic cancer cell proliferation (tumor cells die by apoptosis), angiogenesis and the migration of pancreatic cancer cells[10-14] (Figure 1 and Table 1).

Table 1 Technical features of NK-1 receptor antagonists

NK-1 receptor antagonists	Feature
Therapeutic action	Linked to stereochemical features (receptor affinity) and not to chemical composition
Cell specificity	Specific cytotoxicity against pancreatic cancer cells *via* the NK-1 receptor
Antitumor action	Mitogenesis inhibition Cell death by apoptosis Angiogenesis inhibition Inhibition of the migration of cancer cells: Inhibit invasion, infiltration and metastasis
Beneficial effects	Central nervous system: Antiemetic Anxiolytic Antimigraine Anticonvulsant Neuroprotector Peripheral nervous system: Neuroprotector Liver: Hepatoprotector Kidney: Nephroprotector Systemic: Analgesic Antiinflammatory Antiviral
Side-effects	Headaches, hiccupping, vertigo and drowsiness
Synergistic effect with cytostatic and radiation therapy	Vinblastine, adriamycin, mitomycin, ifosfamide, cisplatin
Decrease cytostatic and radiation therapy side-effects	Cisplatin, cyclophosphamide
Block multiple intracellular signaling pathways	NK-1 receptor (G protein-coupled receptor): Rho-Rock-pMLC: Cell migration inhibition PLC-IP$_3$-Akt: Apoptotic effect PLC-DAG-TK-MAPKs: Inhibition of tumor cell proliferation PLC-DAG-PKC-MAPKs: Inhibition of tumor cell proliferation ATP-cAMP-PKA-Phosphorylation PLA-Arachidonic acid-PGs TXAs LXs Glycogen breakdown inhibition (counteract the Warburg effect)
Dosage	Act at μmol/L in a concentration-dependent manner

Akt: Protein kinase B; ATP: Adenosine triphosphate; cAMP: Cyclic adenosine monophosphate; DAG: Diacilglicerol; IP3: Inositol triphosphate; LXs: Leukotrienes; MAPKs: Mitogen-activated protein kinase; PGs: Prostacyclin; PKA: Protein kinase A; PKC: Protein kinase C; PLA: Phospholipase A; PLC: Phospholipase C; pMLC: Myosin regulatory light chain phosphorylation; TK: Tyrosine-kinase; TXAs: Thromboxanes.

In sum, all the data reported above suggest that novel possibilities for translational research are emerging to improve the diagnosis and treatment of pancreatic cancer. Here, we review the involvement of the SP/NK-1 receptor system in pancreatic cancer and, specifically, the use of NK-1 receptor antagonists as antitumor drugs in pancreatic cancer (Figure 1 and Table 1).

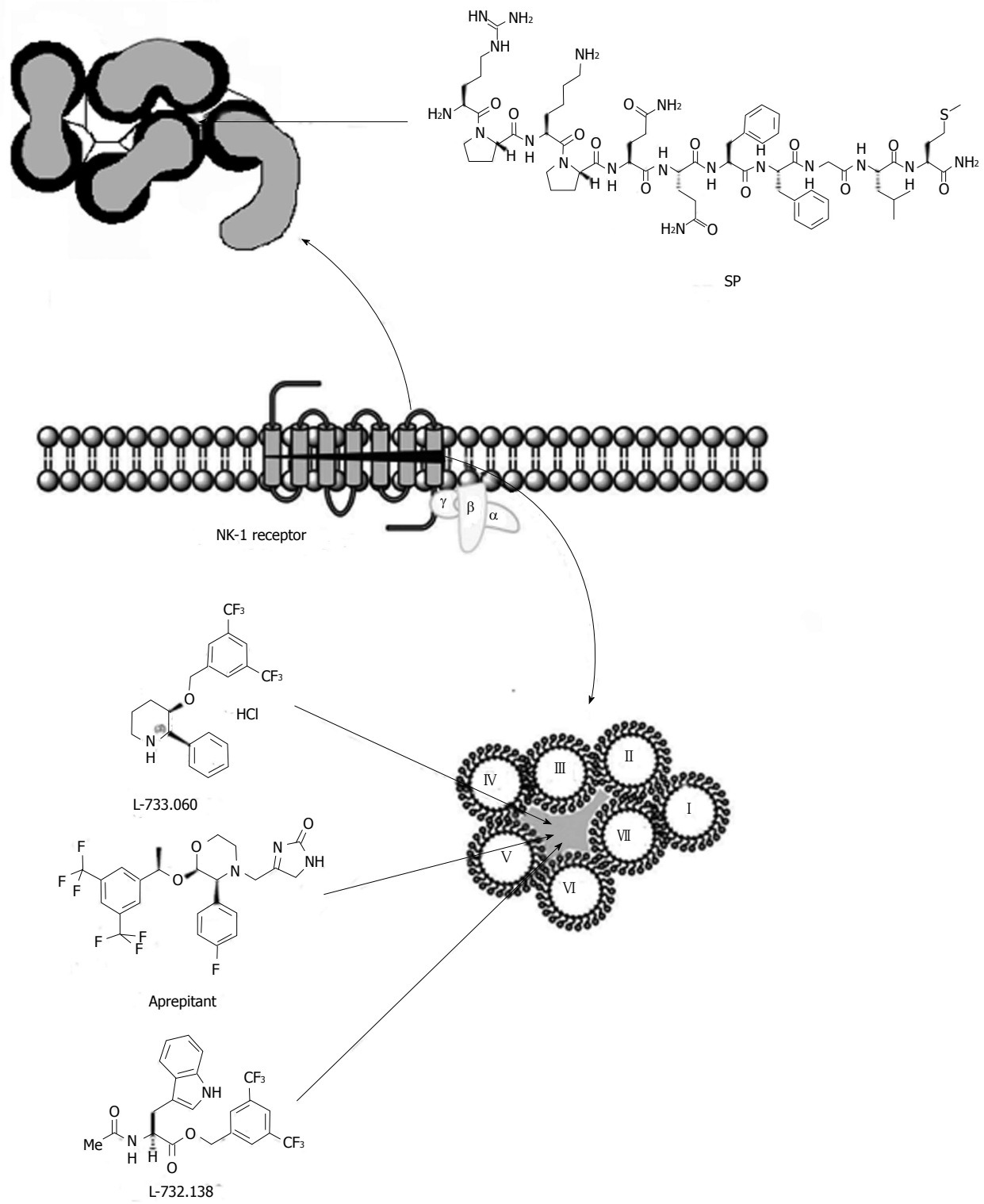

Figure 1 Substance P and neurokinin-1 receptor antagonists bind to different sites of the neurokinin-1 receptor. Substance P (SP) binds to the extracellular loops of the receptor, whereas neurokinin-1 (NK-1) antagonists (*e.g.*, L-733.060, aprepitant, L-732.138) bind more deeply, between the transmembrane segments.

PANCREATIC CANCER CELLS AND SAMPLES EXPRESS NK-1 RECEPTORS

The NK-1 receptor is synonymous with the SP receptor and tachykinin receptor 1. The NK-1 receptor is a G protein-coupled receptor (GPCR) that mediates the action of SP and other tachykinins[15,16]. The NK-1 receptor consists of 407 amino acid residues; it has a molecular weight of 58 kDa, and it is made of seven hydrophobic transmembrane domains with three extracellular and three intracellular loops, an amino-terminus and a cytoplasmic carboxy-terminus[17,18] (Figure 1). The loops have functional sites, including two cysteines amino acids for a disulfide bridge, Asp-Arg-Tyr, which is responsible for

the association with arrestin and, Lys/Arg-Lys/Arg-X-X-Lys/Arg, which interacts with G-proteins[18,19]. The NK-1 receptor is coupled to the Gq family of G proteins and its activation leads to the hydrolysis of membrane phosphoinositides, resulting in the formation of two-second messengers: inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG)[20,21]. The formation of IP3 triggers the release of calcium from intracellular stores and the formation of DAG leads to the activation of protein kinase C. Together, these messengers cause a cascade of protein phosphorylation/dephosphorylation reactions, culminating in altered gene expression and cell function.

SP is an undecapeptide widely distributed throughout the body and it is the natural ligand showing the highest affinity for the NK-1 receptor (Figure 1). In fact, the NK-1 receptor has been defined as a mediator of the biological activities encoded by the C-terminal sequence of tachykinins, for which SP is a more potent agonist than neurokinin A or neurokinin B[22]. After binding to the NK-1 receptor, SP regulates many biological functions (*e.g.*, pain, neurogenic inflammation, mitogenesis...)[4,5], although other NK receptors could also be involved (*e.g.,* NK-2) in these actions. After the binding of SP to the NK-1 receptor, both are internalized into endosomes; the undecapeptide induces a clathrin-dependent internalization of the receptor, after which SP is degraded and the NK-1 receptor is recycled to the cell surface[23-26]. SP-NK-1 receptor binding can generate second messengers [cyclic adenosine monophosphate (cAMP) accumulation *via* stimulation of adenylate cyclase; stimulation, *via* phospholipase C, of phosphatidyl inositol turnover, leading to calcium mobilization; arachidonic acid mobilization *via* phospholipase A2], triggering numerous effectors mechanisms involved in cellular excitability and in the regulation of cell function[4,5,27].

It is known that pancreatic cancer cells and samples express the NK-1 receptor[10,13,14]. This receptor has been also demonstrated in human cancer cell lines and/or in primary tumors (*e.g.*, glioma, astrocytoma, retinoblastoma, ganglioneuroblastoma, leukemia, neuroblastoma, carcinomas (larynx, gastric, colon, medullary thyroid, breast, oral...)[4-6,10,28-37]. In addition, in most tumors investigated NK-1 receptors have been found in intra- and peritumoral blood vessels. This is quite important regarding the involvement of the NK-1 receptor in angiogenesis[6]. NK-1 receptors have been located in both the plasma membrane and the cytoplasm of tumor cells and, occasionally, in the nucleus of these cells[31,34,38]. Moreover, several isoforms (33-38, 46, 54-58 and 75 kDa) of the NK-1 receptor have been reported in human cancer cells (*e.g.*, neuroblastoma, retinoblastoma, larynx carcinoma, gastric adenocarcinoma, leukemia, *etc.*)[33-36,38]. Regarding the pancreatic cancer, it has been reported that its tumor cells express several isoforms (36, 46, 58 and 75 kDa)[10,13,14]. However, in order to clarify the functional roles of these isoforms, further research is needed. In humans, the presence of two subtypes of the NK-1 receptor has been reported: the full-length one and the truncated one. The former mediates a slow growth of tumor cells and the second enhances the growth of these cells to a considerable extent and stimulates the production of cytokines with growth-promoting functions[39]. It seems that these cytokines activate a transcription factor (NF-κB) that upregulates the truncated NK-1 receptor form and slightly increases the full-length form[40,41]. It is also known that the truncated form, an oncogenic isoform of the NK-1 receptor, mediates malignancy in tumor cells[39] and that the truncated NK-1 receptor is increased in colonic epithelial cells from patients with colitis-associated cancer[42].

In the first study in which NK-1 receptors were reported in pancreatic cancer (1 of 9 samples)[6], the authors applied an autoradiographic method. Later, in another study, NK-1 receptor expression was reported in 27% of the samples[43]. However, a third study compared 50-pancreatic human cancer samples obtained from pancreatoduodenoctomy (Whipple operation) with normal controls[10]. In these cases, the authors found the expression of NK-1 receptors in all the pancreatic cancer samples. Thus, by using *in situ* hybridization and immunohistochemistry techniques, in normal pancreas NK-1 receptor mRNA and NK-1 receptor immunoreactivity were occasionally weakly observed in acinar and ductal cells, but a moderate to strong NK-1 receptor mRNA signal and NK-1 receptor immunoreactivity were present in most of the cancer cells[10]. Moreover, the growth of the tumor mass, peritumoral infiltration and metastasis could be regulated by the SP/NK-1 receptor system, overexpressed in tumor cells and in tumoral and peritumoral tissue in pancreatic cancer (inflammatory cells, fibroblasts, blood vessels, nerves, ganglia, islet)[10].

The NK-1 receptor is also known to be involved in the viability of tumor cells. It has been reported that after a knockdown gene-silencing method (siRNA), the NK-1 receptor is involved in the viability of such cells[33,34,37]. Following the administration of the siRNA *TACR1* (tachykinin 1 receptor gene) to cultured tumor cells, more apoptotic cells were found in siRNA cells than in cells not transfected, and hence the number of siRNA tumor cells was significantly decreased in comparison with the number of non-transfected cells[33,34,37].

NK-1 RECEPTOR IS OVEREXPRESSED IN PANCREATIC CANCER CELLS IN COMPARISON WITH NON-TUMOR CELLS

It is known not only that the NK-1 receptor is expressed in tumor cells, but also that this receptor is overexpressed in such cells (*e.g.*, glioblastoma, breast cancer, retinoblastoma, larynx, pancreatic, gastric and colon carcinomas...)[4,5,10,30,33,34,37]. This is important, since the visualization of NK-1 receptors by immunohistochemistry for diagnostic or therapeutic purposes would facilitate the identification of tumors overexpressing this receptor[44]. It is known that normal cells express a lower number of NK-1 receptors than tumor cells (*e.g.*, human pancreatic cancer cell lines express more NK-1 receptors than

control cells)[10]; that tumor samples from patients with advanced tumor stages exhibit significantly higher NK-1 receptor levels[10]; that TACR1 mRNA is present in human acute lymphoblastic leukemia cell lines, with the highest levels in these cells and the lowest ones in normal cells[33]; that astrocytoma/glioma cell lines in culture shows a lower number of NK-1 receptors than astrocytoma/glioma primary tumors; that glioblastomas express more NK-1 receptors than astrocytomas, and that the most malignant phenotypes of tumors show a higher rate of NK-1 receptor expression and are associated with advanced tumor stages and a poorer prognosis[6,10,45]. The data suggest that the number of NK-1 receptors could be correlated with the degree of malignancy. Thus, the overexpression of the NK-1 receptor in tumor cells suggests the possibility of finding a specific treatment against cancer using NK-1 receptor antagonists and, in this way, the side effects of the treatment could be decreased considerably. This strategy opens up new approaches for cancer treatment. Moreover, following the use of real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) methodology in 50 pancreatic human cancer samples obtained from pancreatoduodenoctomy (Whipple operation), NK-1 receptor mRNA levels were increased 36.7-fold in these samples in comparison with normal controls. Enhanced NK-1 receptor expression levels were not related to tumor grade but were associated with advanced tumor stage and a poorer prognosis. As reported above, NK-1 receptor mRNA levels and NK-1 receptor immunoreactivity are higher in human pancreatic cancer samples than in normal pancreas[10]. Moreover, using a Western blot analysis, the NK-1 receptor was found to be increased 26-fold in pancreatic cancer samples in comparison with normal controls. NK-1 receptor mRNA was detected in five pancreatic cancer cell lines by real-time quantitative RT-PCR, the highest levels being observed in CAPAN-1 cells and the lowest ones in ASPC-1 cells. SP and SP analog agonists stimulated pancreatic cancer cell growth, depending on the NK-1 receptor expression level, and this effect could be blocked by a selective NK-1 receptor antagonist in a concentration-dependent manner[10,13].

It has been suggested that chronic inflammation could be correlated with an increased risk of developing cancer. It is known that the risk of pancreatic cancer is very high in subjects with chronic pancreatitis and appears to be independent of sex, country, or type of pancreatitis[46] and that the up-regulation of the NK-1 receptor mRNA expression in chronic pancreatitis has a strong relationship with the pain syndrome that these patients experience[47]. Thus, overexpression of the NK-1 receptor could be involved in chronic pancreatitis-associated cancer. It has also been reported recently that the truncated NK-1 receptor is overexpressed in colonic epithelial cells from patients with colitis-associated cancer, whereas the full-length is not affected[42]. Thus, the overexpression of NK-1 receptors could be used as a diagnostic marker to identify patients at risk of neoplasms and may serve as a useful therapeutic target in the treatment of chronic inflammation-associated cancer.

NANOMOLAR CONCENTRATIONS OF SP INDUCE PANCREATIC CANCER CELL PROLIFERATION AND THE MIGRATION OF TUMOR CELLS

SP acts as a mitogen in normal and tumor cells (*e.g.*, neuroblastoma, astrocytoma, melanoma, retinoblastoma, glioma, melanoma, larynx carcinoma, gastric and colon carcinoma, lymphoblastic leukemia) *via* the NK-1 receptor, since the growth inhibition of many human tumor cells after the administration of NK-1 receptor antagonists is partially reversed by the administration of SP[4,5,33-38,48]. Regarding pancreatic cancer cells, nanomolar SP concentrations elicit the proliferation of the pancreatic cancer CAPAN-1, PA-TU 8902, BxPC-3 and MIA PaCa-2 cell lines[13,14]. By contrast, the mitogenic action of SP on these cell lines could be partially reversed by using NK-1 receptor antagonists such as L-733.060, L-732.138 or the drug aprepitant[12-14]. Many data indicate that SP in a universal mitogen in NK-1 receptor-expressing tumor cells. The undecapeptide can be synthesized and secreted by tumor and non-tumor cells and SP can be released from nerve terminal, and/or it can be released into blood vessels[4,5]. Through these paths, the peptide can exert a mitogenic action on tumor cells. The regulation of local tumor activity through sensory nerves containing SP is relevant, since the undecapeptide could modulate the growth of tumor cells, exerting a direct interaction between the nervous system and the tumor cells. Thus, SP could induce mitogenesis *via* the following mechanisms: (1) autocrine (SP is secreted from tumor cells); (2) paracrine (SP exerts a mitogenic action in endothelial cells); (3) SP is released from nerve terminals; (4) SP reaches the whole body through the bloodstream; this is regulated by the limbic system; and (5) endocrine (SP is released from the tumor mass into the blood vessels)[3-5].

There are multiple cell signaling pathways regulated by SP. After the activation of the NK-1 receptor by SP, an increase in DNA synthesis has been reported in tumor cells, and it seems that *via* the NK-1 receptor the undecapeptide activates members of the mitogen-activated protein kinase (MAPK) family, including extracellular signal-regulated kinases 1 and 2 (ERK1/2) and p38MAPK[45] (Table 1). Once activated, ERK1/2 is translocated into the nucleus, inducing proliferation and protecting the cell from apoptosis[5,7]. In tumor cells, SP increases the phosphorylation and activity of Akt or protein kinase B, a serine-threonine protein kinase that becomes activated *via* phosphatidyl-3-kinase (PI3K); the activation of Akt suppresses apoptosis[49,50]. By contrast, NK-1 receptor antagonists inhibit the basal activity of Akt[51] (Table 1). After it has bound to the NK-1 receptor, other effects are also exerted by SP in tumor cells: it activates phospholipase D and enhances forskolin-stimulated cyclic AMP-production; SP induces the release of interleukins, taurine and

glutamate; it mobilizes intracellular calcium; it induces the formation of inositol phosphate; it stimulates glycogen breakdown; and it influences glutamate and K^+ transport[5,52-56]. The release of interleukins, taurine and glutamate by tumor cells induces an inflammatory process, increasing the levels of SP and hence increasing tumor cell proliferation. Moreover, it has been reported that after binding to the NK-1 receptor SP stimulates glycogen breakdown and increases the intracellular Ca^{2+} concentration in astrocytoma cells. Both effects occur in a concentration-dependent manner. These effects are completely blocked by the NK-1 receptor antagonist CP-96345[55]. In addition, one of the most prominent metabolic alterations in cancer cells is the increase in aerobic glycolysis and the dependency on the glycolytic pathway for adenosine triphosphate generation, known as the Warburg effect, because most cancer cells predominantly produce energy by means of a high rate of glycolysis followed by lactic acid fermentation[57]. Growing tumor cells typically have glycolytic rates up to 200 times higher than those of their normal tissues of origin; this occurs even if oxygen is plentiful. Thus, after binding to the NK-1 receptors located in tumor cells, SP causes glycogen breakdown and the glucose obtained would be used by tumor cells to increase their metabolism[55]. This mechanism could partly explain the Warburg effect. By contrast, NK-1 receptor antagonists block glycogen breakdown in tumor cells[55], and hence can counteract the Warburg effect[3] (Table 1). This new approach to the NK-1 receptor is very interesting because until now the main goal has been the inhibition of the glycolytic enzymes. However, this strategy has not provided any practical results. In cancer treatment, a reduction in glucose formation by blocking the NK-1 receptor may be possible and indeed easier using NK-1 receptor antagonists. Accordingly, without glucose the Warburg effect is not possible in cancer cells.

The migration of tumor cells is a crucial requirement for the development of metastasis and the progression of cancer. At present, over 90% of cancer deaths are derived not from the primary tumor but from the development of metastases[58]. Thus, a major goal in the treatment of cancer should be to inhibit the development of metastases. In this sense, it is known that tumor cell migration is induced by classical neurotransmitters (dopamine, noradrenalin) and peptides (*e.g.*, SP) and that such migration is inhibited after the administration of D_2 receptor, adrenoceptor or NK-1 receptor antagonists[5,59]. It is also known that after binding to the NK-1 receptor SP induces a rapid change in cellular shape (including blebbing) and that membrane blebbing is important in cell movement, cell spreading, and cancer cell infiltration[60,61]. It has recently been reported that SP is involved in pancreatic cancer perineural invasion and that in pancreatic cancer cells SP induces cancer cell proliferation and invasion as well as the expression of matrix metalloproteinase (MMP)-2. SP also promotes neurite outgrowth and the migration of pancreatic cancer cell clusters to the dorsal root ganglia of newborns[14].

NK-1 RECEPTOR ANTAGONISTS INHIBIT PANCREATIC CELL PROLIFERATION IN A CONCENTRATION-DEPENDENT MANNER

At a certain concentration, these antagonists inhibit 100% of tumor cells. NK-1 receptors antagonists are a broad group of heterogeneous chemical compounds (Figure 1 and Table 1). There are two groups: peptide NK-1 receptor antagonists and non-peptide NK-1 receptor antagonists.

Peptide NK-1 receptor antagonists

Most of the work carried out on the design and preparation of antagonists of the NK-1 receptor has focused on the introduction of *D*-amino acids[18]. However, their affinity is several orders of magnitude lower than that of natural agonists, and the metabolic instability of peptide NK-1 receptor antagonists and their inability to gain access to the central nervous system through the blood-brain barrier limit their usefulness for *in vivo* studies. In addition, these substances generally have a number of drawbacks, such as poor potency and a lack of the ability to discriminate between tachykinin receptors, partial residual agonist activity, mast cell degranulating activity, and neurotoxicity after administration in the central nervous system[22]. Some of these peptide NK-1 antagonists are[18]: [D-Arg1, D-Trp 7,9, Leu11] SP (Spantide I). This antagonist is neurotoxic and a potent histamine releaser from mast cells; H-D-Lys (Nicotinoyl)-Pro-[3-(3-pyridyl)-Ala]-pro-D-Phe83,4-Cl2)-Asn-DTrp-Phe-D-Trp-Leu-Nle-NH2 (Spantide Ⅱ). This antagonist is devoid of neurotoxicity; [D-Arg1, D-Trp5, 7, 9, Leu11] SP. This antagonist has anticancer effects in a variety of *in vitro* and *in vivo* models (*e.g.*, pancreatic cancer)[11, 62-65]; (D-Arg1, D-Phe5, D-Trp7, 9, Leu11) SP; (D-Arg1, D-Pro2, D-Trp 7,9, Leu11) SP; [Arg6, D-Trp7,9, MePhe8] SP (6-11); [D-Pro2- Trp7, 9] SP; [D-Pro4, D-Trp7, 9, 10, Phe11] SP (4-11); p-HOPA-DTrp-Phe-DTrp-Leu-Leu-NH2: NY-3238; DMePhe-DTrp-Phe-DTrp-Leu(CH2NH)Leu-NH2: NY-3460.

Non-peptide NK-1 receptor antagonists

Since non-peptide NK-1 receptor antagonists became available[66-68], an increasing number of papers describing new non-peptide antagonists have been published[69]. Thus, steroids (WIN- 51708, *etc.*), perhydroisoindolones (RP-67580, RP-73467, RPR-100.893, *etc.*), benzylamino and benzylether quinuclidine (CP-96345, L-709.210, *etc.*), benzylamino piperidines (CP-99,994, GR-203040, GR-205.171, CP-122.721, *etc.*), benzylether piperidines (L-733.060, L-741.671, L-742.694, *etc.*) and tryptophan based (L-732.138, L-737.488, *etc.*) NK-1 receptor antagonists have been reported[22]. Investigation into non-peptide NK-1 receptor antagonists is a fast-developing field. Some of these peptide NK-1 antagonists have been used in clinical trials and found to be safe. Examples are the drug aprepitant (Figure 1) and its prodrug fosaprepitant, casopitant (GW-679769), vofopitant (GR-205171),

L-759.274, CP-122.721, Ezlopitant (CJ-11.974), Rolapitant, L-754.030, Serlopitant and CJ-11.974[70].

The binding sites for NK-1 receptor antagonists and SP are different[5]. SP is hydrophilic and binds to the extracellular ends of the transmembrane helices and especially to the extracellular loops of the receptor, whereas NK-1 receptor antagonists are lipophilic and bind more deeply between the transmembrane Ⅲ-Ⅶ domains (Figure 1). After binding to the NK- 1 receptor, NK-1 receptor antagonists could block the functions of SP (Table 1). The pharmacologic effect is related to stereochemical features and is not linked to chemical composition. The action is concentration- and time-dependent manner. At higher concentrations, the beneficial effect in the host is summative. Thus, the pharmacologic effects of the NK-1 receptor antagonists are: anxiolytic, antidepressant, antiemetic, antimigraine, antialcohol addiction or neuroprotector effect in the central nervous system, and they also play a role in analgesic, antiinflammatory, and hepatoprotector processes, as well as in antivirus proliferation (Table 1). Regarding cancer, NK-1 receptor antagonists exert an antitumor action (inducing tumor cell death by apoptosis), and they have antiangiogenesis effects and inhibit the migration of tumor cells[3-5] (Table 1). Therefore, the NK-1 receptor antagonists could be considered a new generation of anticancer drugs[3-5,71].

In 1993, Merck initiated studies on NK-1 receptor antagonists based on both CP-96,345 and CP-99,994. L-733.060 (Figure 1) is one of the compounds developed from CP-99,994. It is a 3,5-bistrifluoromethyl benzylether piperidine[72]. The administration of the NK-1 receptor antagonist L-733.060 produces analgesia[73] and antidepressive effects[74,75]. The compound has been suggested for the treatment of anxiety and mood disorders[76] and in inflammatory liver disease, most likely owing to its ability to inhibit the effects of SP[77]. In addition, it has been reported that the NK-1 receptor antagonist L-733.060 acts as an antitumor agent in several human tumor cell lines[13,38,78-81]. In fact, this antitumor action has been reported against pancreatic cancer cell lines[13,14].

A morpholine nucleus that was introduced in the NK-1 receptor antagonist L-742.694 was found to enhance NK-1 receptor-binding affinity[82]. This nucleus was kept in further modifications of the molecule. In order to prevent possible metabolic deactivation, several refinements such as methylation on the C alpha of the benzyl ring and fluorination on the phenyl ring were introduced. These changes afforded the compound MK-869, which showed high affinity for the NK-1 receptor. MK-869 is also called aprepitant (Figure 1) and it has been tested for the treatment of several disorders. Those studies led the Food and Drug Administration to approve the drug Emend, which is indicated for chemotherapy-induced nausea and vomiting and is available for oral use[83]. A water-soluble phosphoryl prodrug for intravenous use, called fosaprepitant, is also available and is marketed as Ivemend[84]. It seems that aprepitant is effective for the treatment of depression[74,75], and it has recently been demonstrated that it is a broad-spectrum antitumor drug[12]. Moreover, the antitumor action of the drug aprepitant against pancreatic cancer cells has been reported. In fact, aprepitant inhibits 100% of pancreatic cancer cells in a concentration-dependent manner[12].

The NK-1 receptor antagonist L-732.138 (*N*-acetyl-*L*-tryptophan 3,5-bis (trifluoromethyl) benzyl ester) (Figure 1) shows a competitive and selective antagonism for the NK-1 receptor. It is approximately 1000-fold more potent in cloned human NK-1 receptors than in cloned human NK-2 and NK-3 receptors, and approximately 200-fold more potent in human NK-1 receptors than in rat NK-1 receptors[85]. The IC_{50} for the human NK-1 receptor expressed in Chinese Hamster Ovary cells is approximately 2.3 nmol[86]. It is known that the administration of L-732.138 produces an attenuation of hyperalgesia[87] and that L-732.138 is able to antagonize H(3) antagonist-induced skin vascular permeability. The antitumor action of the tryptophan-based antagonist L-732.138 against glioma, neuroblastoma and a larynx carcinoma cell lines has been also reported[80], as well as its antitumor action against pancreatic cancer cell lines[13,14].

The immunosuppressive cyclic undecapeptide cyclosporin A (CsA) is a naturally occurring fungal metabolite from *Tolypocladium inflatum Gams*. This molecule has been proposed to play a role in the treatment of human malignancies as an effective modifier of multidrug resistance. It is known that CsA has the pharmacological profile of an NK-1 receptor antagonist[88] and that CsA exerts an antitumor action due to its NK-1 receptor antagonist pharmacological profile in competition assay with SP. The antitumor action of CsA against pancreatic cancer cells occurs in a concentration-dependent manner and pancreatic tumor cells die by apoptosis[89]. However, in clinical practice this interesting therapeutic action of CsA is not possible because the high doses necessary to exert an antitumor action are associated with dangerous side effects, such as kidney failure.

Taking the above data together, it seems that the antitumor action of NK-1 receptor antagonists against pancreatic cancer cells would be due to stereochemical features and that it is not linked to the chemical composition of the antagonists[71] (Table 1), since different compounds (L-733.060, a piperidine derivative; aprepitant, a morpholine derivative; L-732.138, a tryptophane derivative ; CsA, a cyclic undecapeptide) exert an antitumor action (Figure 1). These compounds have only one thing in common: their affinity for the NK-1 receptor.

ANTITUMOR ACTION OF THE NK-1 RECEPTOR ANTAGONISTS IS MEDIATED THROUGH THE NK-1 RECEPTOR AND TUMOR CELLS DIE BY APOPTOSIS

As reported above, the NK-1 receptor antagonists (L-733.060, L-732.138, the drug aprepitant, *etc.*) exert an antitumor action[4,5,33-38] (Figure 1). In particular, these antagonists exert this action against human glioma, larynx

carcinoma, neuroblastoma, rhabdomyosarcoma, leukemia, astrocytoma, osteosarcoma, lymphoma, retinoblastoma, melanoma, lung, breast, and gastric, and colon carcinoma cell lines[4,5,33-38,90,91], as well as against pancreatic cancer cell lines[13,14]. The antitumor action of L-733.060 against human cancer cell lines is more potent than that of aprepitant, and the antitumor action of aprepitant is more potent than that of L-732.138[4,5]. NK-1 receptor antagonists block the SP-induced mitogen stimulation of tumor cells, and they inhibit tumor cell growth in a dose-dependent manner[4,5] (Table 1).

After binding to NK-1 receptors overexpressed in tumor cells, NK-1 receptor antagonists activate the apoptotic machinery and these cells (*e.g.*, pancreatic cancer, *etc.*) die by apoptosis[4,5,12,33,34,38]. Thus, the induction of apoptosis represents a highly suitable approach to cancer treatment, although currently little is known about the mechanisms responsible for the induction of apoptosis in tumor cells. Despite this, it has been reported that the blockade of NK-1 receptors by NK-1 receptor antagonists inhibits the basal kinase activity of Akt. Tumor cells develop strategies to neutralize the multiple pathways leading to cell death, and it has been suggested that one of the most important of these is the expression of the NK-1 receptor[92]. This strategy renders tumor cells highly dependent on the SP stimulus, which provides a potent mitotic signal. This signal could counteract the different death signal pathways activated in tumor cells. The absence of the mitotic signal when the receptor is blocked with NK-1 receptor antagonists could tilt the balance within the cell to favouring apoptotic/death signals, and hence the cell would die[92]. The data reported suggest that NK-1 receptor antagonists could inhibit a large number of tumor cell types in which NK-1 receptors are overexpressed[3-5,33,34,37], and that NK-1 receptor antagonists could be candidates for broad-spectrum antineoplastic drugs including pancreatic cancer[3-5,13,14]. In general, NK-1 receptor antagonists are safe, since the administration of NK-1 receptor antagonists does not induce serious side effects[5,72,93-96], although headaches, hiccupping, vertigo and drowsiness have been reported in humans after their administration[71,95,96] (Table 1). The safety of aprepitant against human fibroblasts has been also demonstrated: the IC_{50} for fibroblasts is three times higher than the IC_{50} for tumor cells[12]. Moreover, the IC_{50} for non-tumor cells is 90 μmol/L but the IC_{100} for tumor cells is 60 μmol/L approximately[12].

Furthermore, it has been reported that the use of chemotherapy and/or radiation therapy and NK-1 receptor antagonists affords a synergistic antitumor action and decreases the side effects of chemotherapy and radiation therapy[5,97,98] (Table 1). Furthermore, it has been suggested that the co-administration of NK-1 receptor antagonists and microtubule-destabilizing agents (*e.g.*, vinblastine) could be useful in cancer, since these compounds have a synergic effect[5,98] (Table 1). This combination is synergistic for the growth inhibition of NK-1 receptor-possessing cancer cells, but not for normal cells. A better understanding of the mechanisms underlying this interaction is needed in order to assess the clinical relevance of this novel synergistic combination. Moreover, synergism has been reported for the combination of L-733.060 with common cytostatic drugs (adriamycin, mitomycin, ifosfamide, cisplatin) in MG-63 human osteosarcoma cells, but not in non-malignant HEK293 cells[99]. Pretreatment of HEK293 with L-733.060 prior to exposure to cytostatic drugs partially protected HEK293 cells from inhibition by these drugs[99].

NK-1 RECEPTOR ANTAGONISTS INHIBIT ANGIOGENESIS IN PANCREATIC CANCER XENOGRAFTS

Neovascularization or neoangiogenesis is a sequential process, with early endothelial proliferation followed by new vessel formation and increased blood flow, accompanied by maturation of endogenous neurovascular regulatory systems occurring late in this process in inflamed tissues[100]. The growth of new vessels from a pre-existing vasculature is a common feature of chronic inflammation (early neoangiogenesis is a key step in the transition from acute to persistent inflammation) and wound healing. Neoangiogenesis, a hallmark of tumor development, has also been associated with increased tissue innervation and the expression of NK-1 receptors. In a large majority of tumors investigated, SP and NK-1 receptors are found in the intra and peritumor blood vessels[6]. These findings have been reported specifically in pancreatic cancer[10]. SP, a main mediator of neurogenic inflammation through the release of the peptide from peripheral nerve terminals, is involved in the growth of capillary vessels *in vivo* and in the proliferation of cultured endothelial cells *in vitro*. Additionally, it is known that the proliferation of endothelial cells by NK-1 receptor agonists (SP or SP analog agonists) increases in a concentration-dependent manner (NK-1 receptor antagonists block the proliferative action of SP), whereas the action of selective NK-2 and NK-3 receptor agonists has no significant effects on the proliferation of endothelial cells. These findings indicate that NK-1 receptor agonists (*e.g.*, SP) can stimulate the process of neovascularization directly, probably through the induction of endothelial cell proliferation[101], and that SP enhanced angiogenesis results from a direct action on microvascular NK-1 receptors. Thus, through such receptors found at high density in blood vessels SP may strongly influence vascular structure and function inside and around tumors by increasing tumor blood flow and by fostering stromal development[6]. By contrast, it has been reported that NK-1 receptor antagonists inhibit endothelial cell proliferation and angiogenesis in a concentration-dependent manner[101] (Table 1). It has also been reported that the [D-Arg1, D-Trp5,7,9, Leu11] SP analog antagonist (SPA, broad-spectrum GPCR antagonist, peptide NK-1 receptor antagonist) has an antitumor action[11]. It is known

that in ductal pancreatic cancer cells expressing NK-1 receptors, NK-1 receptor antagonists induce the synthesis of proangiogenic chemokines and that in HPAF-Ⅱ, a well-differentiated pancreatic cancer cell line, peptide NK-1 receptor antagonists inhibit Ca^{2+} mobilization and DNA synthesis[11]. These antagonists also significantly attenuated the growth of HPAF-Ⅱ tumor xenografts in nude mice beyond the treatment period. Interestingly, one peptide NK-1 receptor antagonist (SPA, broad-spectrum GPCR antagonist) markedly increases apoptosis but moderately decreases the proliferation marker Ki-67 in tumor xenografts, implying additional mechanisms for the significant growth inhibitory effect[11]. HPAF-Ⅱ cells express ELR^+ CXC chemokines, including interleukin-8/CXCL8, which bind to CXCR2 (a member of the GPCR superfamily) and promote angiogenesis in many types of cancer, including pancreatic cancer. A salient feature of these results is that peptide NK-1 receptor antagonists markedly reduced tumor-associated angiogenesis in HPAF-Ⅱ xenografts *in vivo*. The data suggest that peptide NK-1 receptor antagonists (SPA, broad-spectrum GPCR antagonist) attenuate tumor growth in pancreatic cancer *via* a dual mechanism involving both antiproliferative and antiangiogenic properties[11]. Thus, the dual-inhibitory effect of peptide NK-1 receptor antagonists could be of significant therapeutic value in pancreatic cancer, when used in combination with other anticancer drugs. In sum, all these data indicate that the SP/NK-1 receptor system controls neoangiogenesis in pancreatic cancer and that, in addition, this system could also regulate the growth of the pancreatic tumoral mass, since NK-1 receptors are overexpressed in tumoral cells and in peritumoral pancreatic cancer tissues[10]. Thus, by using NK-1 receptor antagonists (peptide or non-peptide), the NK-1 receptor could be used as a target to inhibit both neoangiogenesis and the growth of pancreatic cancer (Figure 1 and Table 1).

Accordingly, targeted therapies for pancreatic cancer offer new ways to search for potentially more effective strategies. Thus, the use of NK-1 receptor antagonists in chronic pancreatitis could: (1) improve chronic inflammation; (2) improve pain; and (3) prevent the chronic pancreatitis associated with cancer. The use of NK-1 receptor antagonists in pancreatic cancer could exert: (1) an antitumor action, by inhibiting pancreatic cancer cell proliferation (tumor cells die by apoptosis); (2) antiangiogenic properties; and (3) inhibition of the migration of pancreatic cancer cells (preventing invasion, infiltration and metastasis). Thus, the antitumor action of NK-1 receptor antagonists in pancreatic cancer could be specifically for a single target: the NK-1 receptor (Figure 1). The mechanisms of action of NK-1 receptor antagonists are the opposite of those involved in classic chemotherapy. In addition, NK-1 receptor antagonists not only exert an antitumor action, but also elicit beneficial effects in the host such as anti-inflammatory, analgesic, anxiolytic, antidepressant, antiemetic, hepatoprotector and neuroprotector effects[4,5] (Table 1).

SAFETY OF NK-1 RECEPTOR ANTAGONISTS IN HUMAN CLINICAL TRIALS

As reported above, an upregulation of the SP/NK-1 receptor system occurs in human pancreatic cancer cells and hence the NK-1 receptor can be considered as an important target for the treatment of this disease. The overexpression of the NK-1 receptor in human pancreatic cancer cells suggests that the administration of NK-1 receptor antagonists is an excellent strategy for the treatment of this disease (these antagonists, after binding to NK-1 receptors, induce the apoptosis of tumor cells) and in addition fewer side effects should be expected after the administration of these drugs to patients, since NK-1 receptor antagonists are specific for a determined target, the NK-1 receptor, which is overexpressed in cancer cells and it is involved in the viability of tumor cells[3]. It should be noted that the IC_{100} for cancer cells is 60 μmol/L approximately but the IC_{50} for non-tumor cells is 90 μmol/L[12].

Many studies have reported the absence of serious side effects when non-peptide NK-1 receptor antagonists have been administered to humans[71]. It is known that the NK-1 receptor antagonist GR-205171 alleviated anxious symptoms in patients with social phobia[102]. Several non-peptide NK-1 receptor antagonists (*e.g.*, casopitant, orvepitant, vestipitant, vofopitant) have been also tested in human clinical trials for the treatment of depression, anxiety disorders, post-traumatic stress disorder, alcoholism, panic disorder and schizophrenia[103,104]. In some trials, these antagonists exerted an anxiolytic or an antidepressant action and in all the cases showed a low side effect profile. Moreover, the analgesic action of the NK-1 receptor antagonists aprepitant, lanepitant (LY-303870), AV-608 and CJ-11.974 has been tested in human trials and in all the cases the drug was ineffective in relieving pain (*e.g.*, neuropathic pain, visceral pain, osteoarthritis, fibromyalgia)[105]. However, the NK-1 receptor antagonist CP-99994 decreased postoperative dental pain[106]. NK-1 receptor antagonists have been also tested for the treatment of migraine. Thus, lanepitant was ineffective in migraine prevention and acute migraine; RPR-100.893 had no effects on migraine attacks; L-758.298 failed to abort migraine attacks, and GR-205171 was ineffective against the treatment of migraine[106]. Moreover, it has been reported that HIV-infected adults not receiving antiretroviral therapy, low (125 mg) and high (250 mg) doses of aprepitant (daily, for 14 d) were found to be safe[107]. Neurological adverse events (headache, hypersomnia, lightheadedness, dizziness) were observed in the 50% of the patients that received a higher dose of the NK-1 receptor antagonist, whereas insomnia was reported in those treated with 125 mg of aprepitant (11.1% patients). In both groups, the concentration of SP in plasma decreased. Gastrointestinal, ocular/visual, dermatological and systemic adverse events were also reported in the patients treated with aprepitant[107]. No changes in sleep

quality, anxious mood, depressed mood or neurocognitive measures were found[108].

Despite the large number of non-peptide NK-1 receptor antagonists reported, the only NK-1 receptor antagonist used currently in clinical practice is the drug aprepitant (Emend, MK-869, L-754.030) (oral) and its intravenously administered prodrug, fosaprepitant[3]. Fosaprepitant is rapidly converted to aprepitant *via* the action of ubiquitous phosphatases[108]. Both NK-1 receptor antagonists are used for the prevention of chemotherapy-induced nausea and vomiting and post-operative nausea and vomiting[70]. Many clinical human trials have reported the efficacy and safety of aprepitant/fosaprepitant for the treatment of emesis[70]. No serious adverse events were found. Aprepitant was well tolerated: no grade 3 or higher toxicities related to aprepitant were reported, whereas the adverse events mostly observed were fatigue, diarrhoea, febrile neutropenia, headache, dyspnea, constipation and hiccups[109].

Accordingly, novel possibilities for translational research are emerging for improving the treatment of diseases in which the SP/NK-1 receptor system is upregulated and hence, in particular, the use of NK-1 receptor antagonists in oncology therapy is quite promising according to the data obtained from preclinical studies[3]. Aprepitant is an excellent candidate for testing its antitumor, antimigratory and antiangiogenic action in human clinical trials since a large part of the required safety and characterization studies for aprepitant have already been carried out (aprepitant is already available in clinical practice for the treatment of emesis)[70]. Moreover, aprepitant has been developed as a nanoparticle formulation to enhance exposure and to minimize food effects. In humans, the nanoparticle formulation increased 3 times-4 times the bioavailability of this NK-1 receptor antagonist[110]. It has been also demonstrated in an *in vivo* study that fosaprepitant reduced significantly the tumor volume of MG-63 human osteosarcoma xenografts[99].

It seems that by increasing the number of days on which aprepitant is currently administered and using higher doses of aprepitant than those used in chemotherapy-induced nausea and vomiting this NK-1 receptor antagonist could be effective in cancer (*e.g.*, pancreatic cancer)[3]. However, these issues should be investigate in depth. By increasing the dose of aprepitant, higher and undescribed side effects may occur, although it has been reported that in patients with depression a dose of 300 mg/d of aprepitant was well tolerated and no significant difference in the frequency of adverse events was observed as compared with placebo[3].

CONCLUSION

The SP/NK-1 receptor system plays an important role in the development of pancreatic cancer, neoangiogenesis and metastasis. It seems that SP acts as a mitogen for pancreatic tumor cells overexpressing NK-1 receptors and that NK-1 receptor antagonists also induce apoptosis in tumor cells. Research into the involvement of the SP/NK-1 receptor system in pancreatic cancer must continue in forthcoming years since it is necessary to explore new and effective therapeutic interventions in pancreatic cancer research. It is important to seek strategies targeting tumor-specific molecular derangements. This is the case of the NK-1 receptor, which is overexpressed in pancreatic tumor cells and tumor samples. NK-1 receptor antagonists induce the death of tumor cells by apoptosis. Accordingly, the NK-1 receptor is a promising target in the treatment of pancreatic cancer and NK-1 receptor antagonists could be considered as drugs for the treatment of this tumor. This conclusion is based on the following data: (1) after binding to the NK-1 receptor, SP induces pancreatic tumor cell proliferation, angiogenesis and the migration of pancreatic tumor cells (invasion, infiltration and metastasis); and (2) by contrast, NK-1 receptor antagonists inhibit pancreatic tumor cell proliferation (tumor cells die by apoptosis), have antiangiogenic properties in pancreatic cancer, and block the migratory activity of pancreatic tumor cells. Currently, in clinical practice there are few new drugs against the treatment of pancreatic cancer. However, it has been demonstrated *in vitro* and *in vivo* that NK-1 receptor antagonists exert an antitumor activity against pancreatic cancer cells. At the present, there are more than 300 NK-1 receptor antagonists[69] and this means that there are more than 300 potential drugs against the treatment of pancreatic cancer. Thus, it is crucial to test the antitumor action of NK-1 receptor antagonists in human clinical trials. In this sense, the antitumor action of NK-1 receptor antagonists already available in clinical practice for the treatment of emesis (*e.g.*, aprepitant) should be tested in clinical trials. It has previously been reported that the administration of aprepitant is well tolerated and is associated with minimal side effects. Indeed, at 300 mg/d of aprepitant was well tolerated and no significant difference in the frequency of adverse events were observed in comparison with placebo administration[71]. It is also known that, *in vitro*, aprepitant exerts an antitumor action against human pancreatic tumor cells[12]. In sum, all the data point to the notion that the NK-1 receptor could be a new and promising therapeutic target in pancreatic cancer and that NK-1 receptor antagonists could open the door to a new and promising generation of anticancer drugs against pancreatic cancer.

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TOPIC HIGHLIGHT

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Hedgehog signaling pathway as a new therapeutic target in pancreatic cancer

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Abstract

Pancreatic cancer is one of the most aggressive and difficult cancers to treat. Despite numerous research efforts, limited success has been achieved in the therapeutic management of patients with this disease. In the current review, we focus on one component of morphogenesis signaling, Hedgehog (Hh), with the aim of developing novel, effective therapies for the treatment of pancreatic cancer. Hh signaling contributes to the induction of a malignant phenotype in pancreatic cancer and is responsible for maintaining pancreatic cancer stem cells. In addition, we propose a novel concept linking Hh signaling and tumor hypoxic conditions, and discuss the effects of Hh inhibitors in clinical trials. The Hh signaling pathway may represent a potential therapeutic target for patients with refractory pancreatic cancer.

Key words: Hedgehog signaling pathway; Pancreatic cancer; Cancer stem cells; Hypoxic condition; Therapeutic target

Core tip: Hedgehog (Hh) signaling is involved in the induction of malignant potential in pancreatic cancer, controlling processes of proliferation, invasiveness and tumorigenesis. This phenotypic change is closely associated with the nuclear factor kappa-light-chain-enhancer of activated B cells transcription factor, both in an autocrine and paracrine manner. Hh signaling is also capable of maintaining pancreatic cancer stem cells, and may be activated under conditions of tumor hypoxia. Thus, the Hh signaling pathway may represent a potential therapeutic target for patients with refractory pancreatic cancer and the use of Hh inhibitors will likely play an important role in future therapeutic strategies.

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INTRODUCTION

Pancreatic cancer remains one of the deadliest cancers, with an overall survival rate of < 5%[1]. An underlying reason for this may be that few patients undergo curative, surgical operations because of the advanced stage of the cancer at the time of diagnosis. Furthermore, apart from chemotherapy and radiation therapy, there are no effective, alternative therapies for the treatment of refractory pancreatic cancer, and as such, the development of novel therapeutic strategies is urgently required. Recently, it was shown that the Hedgehog (Hh) signaling pathway, which plays a key role in morphogenesis signaling, is re-activated in pancreatic cancer[2]. Hh signaling contributes to tumor aggressiveness, affecting key tumorigenic processes such as proliferation, invasion

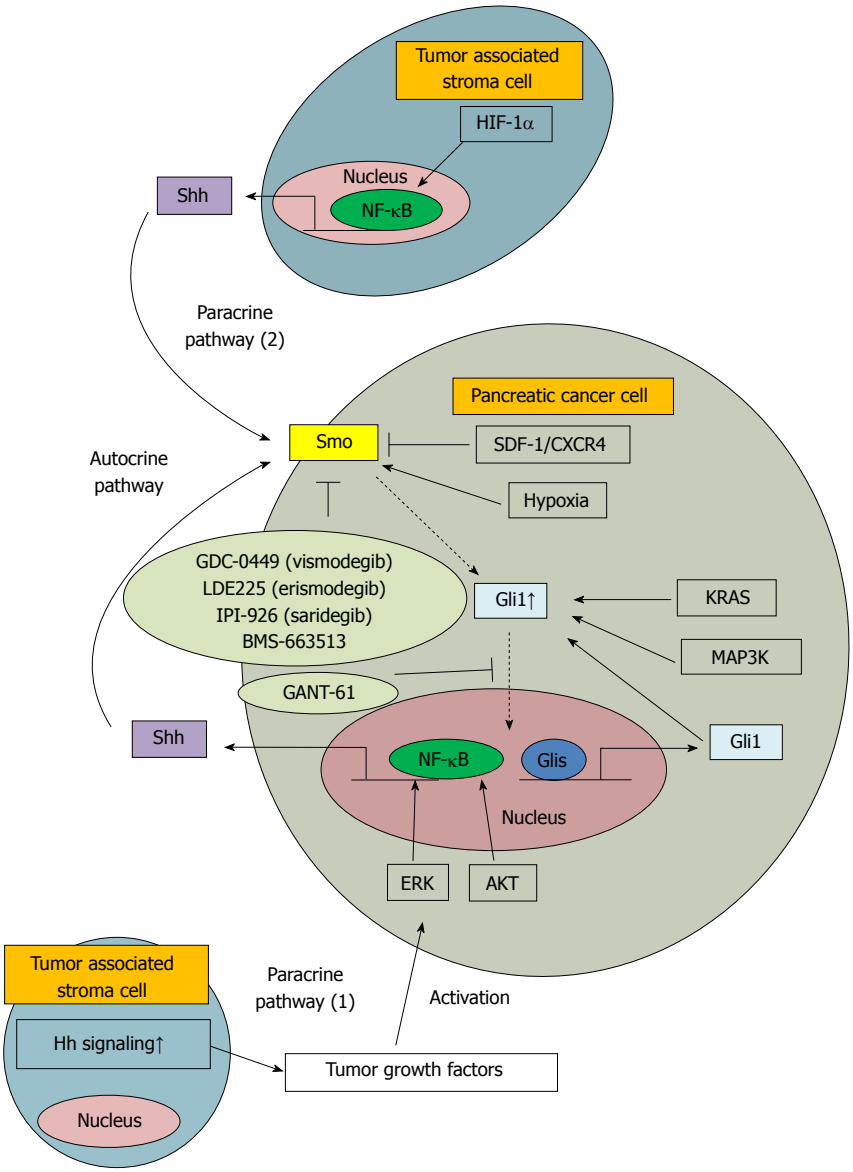

Figure 1 Schematic review. Hedgehog (Hh) signaling is activated in both autocrine and paracrine pathways. Tumor associated stroma cells play a pivotal role in tumor progression related to activation of Hh signaling [paracrine pathways (1) and (2)]. Induction of sonic Hh (Shh) is closely associated with activation of the nuclear factor kappa-light-chain-enhancer of activated B cells transcription factor in pancreatic cancer. Shh is produced by NF-κB activation in pancreatic cancer cells and tumor associated stroma cells. Pathways contributing to Smo and Gli1 activation include SDF-1/CXCR4, hypoxia, KRAS and MAP3K. The effects of Hh inhibitors including GDC-0449 (vismodegib), LDE225 (erismodegib), IPI-926 (saridegib), BMS-663513 and GANT-61 in clinical trials are under investigation. Dotted arrows show components of the Hh signaling pathway in tumor cells focused on in this review.

and progression of cancer cells. Therefore, inhibitors targeting Hh signaling have drawn significant attention as novel, molecularly targeted drugs. Hh signaling components including Patched and Smoothened (Smo) have been detected in almost 70% of human pancreatic cancer specimens and consequently, Hh signaling may play a critical role in the genesis of pancreatic cancer cells[2]. In this review, we summarize recent efforts in the development of new, therapeutic strategies to treat pancreatic cancer, targeting the Hh signaling pathway.

HH SIGNALING PATHWAY

The Hh signaling pathway plays a pivotal role in embryonic patterning and growth control, acting as a morphogen, mitogen and inducing factor of developing organs[3-7]. Hh signaling normally ceases after embryogenesis, however in various cancers, including pancreatic cancer, Hh signaling is re-activated[8]. Therefore, the regulation of Hh signaling in pancreatic cancer likely plays important role in tumorigenesis. The Hh signaling pathway is composed of Hh proteins (sonic Hh; Shh, Indian Hh and Desert Hh), the 12-transmembrane Patched proteins (Patched 1 and Patched 2), the 7-transmembrane protein, Smo and the 5-zinc-finger transcription factors, Gli1, Gli2 and Gli3[9-11]. In the absence of Hh ligand, Patched suppresses Smo, which is the driving protein for Hh signaling, and Gli2 and Gli3 are cleaved

by ubiquitin ligases to generate transcriptional repressor isoforms[12-14]. In contrast, in the presence of Hh ligand, inhibition of Smo by Patched is released, Smo is activated, and Gli2 and Gli3 are transmitted to the nucleus as full-length activators leading to the transcription of target genes such as Patched and Gli1[12-14]. Recent studies demonstrated the existence of primary cilia on the cell surface and showed that Smo moves from the cytoplasm to primary cilia in the process of activation[15]. One of the target genes of Hh signaling; Ptch and Gli1 regulate the transcription of the Hh responsive genes by themselves[16]. Other target genes of Hh signaling are the cell cycle regulator Cyclin D1, p21 and N-Myc which plays important role for carcinogenesis and is also typically dysregulated in the cancer cells[7,17,18]. The Hh signaling pathway is unique because several components of this pathway consist of both oncogenes and cancer suppressor genes.

HH SIGNALING AND THE INDUCTION OF MALIGNANT POTENTIAL IN PANCREATIC CANCER

Originally, the relationship between Hh signaling and tumorigenesis was reported following the association of mutations in genes such as *Gli1*, *Patch* and *Smo* in glioblastoma, basal cell carcinoma and rhabdomyosarcoma[19-21]. In pancreatic cancer, ligand-dependent activation of Hh signaling, but not genomic mutation, was first reported[2]. Previous studies have also shown that Shh overexpression is sufficient to initiate pancreatic intraepithelial neoplasia (PanIN)-like precursor lesions[2,22]. At present, this ligand-dependent pathway is thought to be the major mechanism underlying Hh signaling activation. Two distinct ligand-dependent activation pathways exist; autocrine and paracrine. In addition, association between chronic inflammation and the development of cancer has been recognized for several years[23-27]. In both autocrine and paracrine pathways, NF-κB plays a pivotal role. NF-κB is a transcription factor that controls expression of numerous genes involved in inflammation and immune response processes, including proliferation, invasion, adhesion, angiogenesis and apoptosis[28]. In the autocrine pathway, Shh is a direct transcriptional target of NF-κB, and proliferation of pancreatic cancer cells is accelerated *via* overexpression of Shh[29,30]. In the paracrine paradigm, tumor-associated stroma is important as a microenvironmental factor[31,32]. In one paracrine pathway, stroma cells surrounding pancreatic ductal adenocarcinoma cells, secrete tumor-growth factors through stromal Hh signaling activation[31]. This may explain why low concentrations of Hh signaling antagonist are sufficient to inhibit tumor growth [paracrine pathway (1), Figure 1][31]. In an alternative paracrine pathway, NF-κB-activated monocytes located in the tumor stromal area produce Shh, which stimulates the Hh signaling pathway in pancreatic cancer [paracrine pathway (2), Figure 1][33]. Inhibition of Hh signaling targets pancreatic stellate cells in the tumor-associated stroma, specifically reducing pancreatic tumor growth and metastasis[34,35]. In addition, Singh *et al*[36] showed that CXCL12/CXCR4 protein signaling induces Shh expression in pancreatic cancer *via* extracellular regulated kinase (ERK) and Akt kinase-mediated activation of NF-κB. Some other molecules affected by the activation of Hh signaling may also contribute to the induction of malignant potential in pancreatic cancer. Decrease in Cyclin D1by the inhibition of Hh signaling induces the G_0/G_1 arrest and inhibits cell proliferation[37]. Matrix metalloproteinase (MMP)-9 and MMP-2 locate the downstream of Gli1 and are involved with the invasiveness in pancreatic cancer[38,39].

HH SIGNALING AND PANCREATIC CANCER STEM CELLS

Solid tumor cancer stem cells were first identified in breast cancer as $CD24^{-/low}CD44^{+}$ cells[40]. $CD44^{+}CD24^{+}$epithelial-specific antigen $(ESA)^{+}$ pancreatic cancer cells are reported to exhibit the stem cell characteristics of self-renewal and the ability to produce differentiated progeny[41]. Most importantly, cancer stem cells (CSCs) are characterized by features of resistance towards conventional chemotherapy and radiotherapy[42-45]. Pancreatic CSCs exhibit upregulation of Shh[46]. Recently, inhibition of Hh signaling was reported to inhibit the self-renewal of pancreatic CSCs and reverse chemoresistance[47]. Subsequent studies demonstrated that various agents were capable of inhibiting pancreatic CSCs *via* suppression of Hh signaling. For example, Tang *et al*[48] revealed that epigallocatechin-3-gallate, an active compound in green tea, inhibits the self-renewal capacity of pancreatic CSCs *via* inhibition of Hh signaling components including Smo, Ptch, Gli1 and Gli2. Other groups demonstrated that sulforaphane, a component of dietary cruciferous vegetables, decreases pancreatic CSC self-renewal *via* inhibition of Hh signaling components, Smo, Gli1 and Gli2[49,50]. Han *et al*[51] has revealed that suppression of Hh signaling by arsenic trioxide leads to the inhibition of the viability of pancreatic CSCs using animal models. A better understanding of the molecular pathways driving CSCs will lead to the development of effective, new therapeutic approaches for the treatment of pancreatic cancer.

As previously discussed, there are numerous reports describing $CD44^{+}CD24^{+}$ double positive cells in pancreatic CSCs. However to date, there have been relatively few studies investigating CD24 or CD44 molecules alone as therapeutic targets in pancreatic CSCs. CD24 is a unique molecule because it is described as a marker of pancreatic CSCs, whereas it is expressed at low levels or is absent in breast CSCs. CD24 is thought to act as an adhesion molecule[52,53]. Recently, truncated Gli1 was shown to induce clinically more aggressive cancer *via* the increased expression of CD24[54]. Ringel *et al*[55] showed that constitutive expression of CD44 variants may also be associated with

the malignant state of invasive pancreatic carcinoma. However the precise roles CD24 and CD44 in pancreatic CSCs remain unclear.

HH SIGNALING AND HYPOXIA

Pancreatic cancer is thought to occur under high levels of hypoxia[56]. Therefore, a detailed understanding of the hypoxic microenvironment is crucial for developing effective therapeutic approaches to treat this malignancy. Previous studies have shown that the oxygen concentration in venous blood and deep tumor environments is 5.3% and 1.3%, respectively[57,58]. Thus, to accurately analyze the molecular mechanisms underlying pancreatic cancer, experiments performed under hypoxic conditions are required. The relationship between hypoxia and Hh signaling activation was first reported in 2011, with a study showing that hypoxia activates Hh signaling pathway by upregulating Smo transcription[38]. Thereafter, it was reported that hypoxia induces epithelial to mesenchymal transition (EMT) *via* activation of Hh signaling[59]. Interestingly, under hypoxic conditions, activation of Hh signaling is independent of hypoxia inducible factor (HIF)-1α and is also ligand-independent, with no observable increase in Shh[38,59]. Conversely, Spivak-Kroizman *et al*[60] showed that hypoxia and desmoplasia led to more aggressive and therapy-resistant tumors *via* activation of Hh signaling by Shh, due to HIF-1α activation in the stroma. The mechanisms underlying activation of Hh signaling under hypoxic conditions remains unclear. However, given that Hh signaling is activated under tumor hypoxic conditions, this pathway may represent an important therapeutic target. Indeed, protein-bound polysaccharide decreases invasiveness and proliferation in pancreatic cancer by inhibition of Hh signaling, especially under hypoxia[39].

HH SIGNALING AND THERAPEUTIC APPROACHES IN PANCREATIC CANCER

Pancreatic cancer is often refractory to standard treatments, and many patients are unable to undergo surgery because of the advanced stage of disease at the time of diagnosis. Chemotherapy using gemcitabine and 5-FU derivatives, Tegafur-Gimeracil-Oteracil Potassium (S-1), are often used in Japan. However, combined use of Hh inhibitors with gemcitabine or 5-FU may induce chemoresistance[37]. One reason may be that gemcitabine and 5-FU are sensitive to S-phase and that Hh inhibitor often induces G_1 arrest in cancer cells[37]. Conversely, several groups have shown that combined treatment with Hh inhibitors and gemcitabine has a synergistic effect on tumor growth in a xenograft model[61]. Combined use of Hh inhibitors and cisplatin, a cell cycle independent drug, may also have a synergistic effect[37]. Molecular targeting drug is now well established and the combined use of Hh inhibitors and other targeted drugs is currently being studied and utilized. For example, there is a possible synergistic relationship between Hh and epidermal growth factor receptor (EGFR) signaling pathways in pancreatic cancer[62-64]. Although combination therapy with Hh inhibitors remains controversial, these findings will be essential for developing new effective therapeutic strategies. Radiation is considered the third therapeutic strategy for the treatment of pancreatic cancer. Recently, focal radiation in combination with Hh inhibitors exhibited synergistic effects on reducing lymph node metastasis in pancreatic cancer[65]. Immunotherapy is anticipated as the fourth line of therapy after surgery, chemotherapy and radiation. In this approach, activated lymphocytes and dendritic cells (DCs) derived from patients with advanced cancer are often used. Recently, it was reported that Hh signaling is revitalized in activated lymphocytes and DCs derived from patients with advanced cancer and used for immunotherapy, and that this plays a pivotal role in the maintenance of their functions[66,67]. Therefore, Hh inhibitors may not have a synergistic effect when combined with immunotherapy.

Within the class of Hh inhibitors, recent drug development has focused on Smo inhibitors. Although exact patients' outcome has not been reported yet, Sekulic *et al*[68] has shown that the independently assessed response rate was 30% and 43%, and the median duration of response was 7.6 mo using two-cohort study with GDC-0449 (vismodegib) in metastatic and locally advanced basal-cell carcinoma. GDC-0449 and IPI-926 (saridegib) are currently under phase Ⅱ clinical trials in metastatic, advanced and recurrent pancreatic cancer[69] and BMS-663513 is under phase I clinical trial[70]. A recent study demonstrated that LDE225 (erismodegib), a Smo antagonist, suppresses tumor growth and prolongs survival in a murine model of islet cell neoplasms[71]. Furthermore, GANT-61, a Gli transcription factor inhibitor, has been shown to inhibit pancreatic cancer stem cell growth[72]. An overview of Hh signaling inhibitors is shown in Figure 1. More recently, inhibition of Hh signaling has received significant attention as an anti-tumor strategy. Based on this, the relationship between Hh signaling and various materials has been reported. For instance, resveratrol, 3,4',5-trihydroxystilbene inhibits proliferation and induces apoptosis *via* Hh signaling in pancreatic cancer[73]. Curcumin, a phenolic compound extracted from Zigiberaceae turmeric, reverses EMT of pancreatic cancer by inhibiting Hh signaling[74]. Triparanol, a known cholesterol biosynthesis inhibitor blocking the 24-dehydrocholesterol reductase, suppresses pancreatic cancer tumor growth by deregulation of Hh signaling[75].

Gli1 is both a transcription factor and a target gene, as shown in previous reviews, and crosstalk between Hh signaling and other pathways has been demonstrated[8]. Gli1 is activated *via* several kinds of signaling pathways. In pancreatic cancer, various signaling pathways including KRAS[76], ERK[36], AKT[36], MAP3K[77] and SDF-1/CXCR4[78] are associated with Hh signaling (Figure 1). Because Gli1 is located downstream in many of these

pathways, it may represent a better therapeutic target.

CONCLUSION

In this review, we have summarized the development of pancreatic cancer treatment, with specific focus on the Hh signaling pathway. The Hh signaling pathway may represent an important therapeutic target in pancreatic cancer because this pathway is activated in the majority of pancreatic cancers and both ligand-dependent and independent inhibitors are effective. Hh inhibitor can successfully inhibit tumor growth and invasiveness *in vitro* and can be a promising drug, however, in clinical trial, it is not easy to verify the effectiveness of Hh signaling inhibitor. This reason may be that the actual function of Hh signaling molecules are not fully understood[79,80].

Hh signaling inhibitors should be effective in cancers in which Hh components are mutated such as basal cell carcinoma, basal cell nevus syndrome and medulloblastoma because Hh signaling is constitutively activated[81]. And in these cancers, Hh signaling inhibitors may become the first use drug in future clinical life. However, for other tumors, appropriate combination therapy may be required for the effective therapy. In January 2012, the Smo inhibitor, vismodegib, was clinically approved for the first time by the US Food and Drug Administration, for the treatment of unresectable or metastatic basal cell carcinomas of the skin[82]. Hh signaling inhibitors will now be used in pancreatic cancer as a monotherapy and in combination therapy with other chemodrugs, molecularly targeted drugs or radiation therapy.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Minimally invasive radical pancreatectomy for left-sided pancreatic cancer: Current status and future perspectives

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Abstract

Minimally invasive distal pancreatectomy with splenectomy has been regarded as a safe and effective treatment for benign and borderline malignant pancreatic lesions. However, its application for left-sided pancreatic cancer is still being debated. The clinical evidence for radical antegrade modular pancreatosplenectomy (RAMPS)-based minimally invasive approaches for left-sided pancreatic cancer was reviewed. Potential indications and surgical concepts for minimally invasive RAMPS were suggested. Despite the limited clinical evidence for minimally invasive distal pancreatectomy in left-sided pancreatic cancer, the currently available clinical evidence supports the use of laparoscopic distal pancreatectomy under oncologic principles in well-selected left sided pancreatic cancers. A pancreas-confined tumor with an intact fascia layer between the pancreas and left adrenal gland/kidney positioned more than 1 or 2 cm away from the celiac axis is thought to constitute a good condition for the use of margin-negative minimally invasive RAMPS. The use of minimally invasive (laparoscopic or robotic) anterior RAMPS is feasible and safe for margin-negative resection in well-selected left-sided pancreatic cancer. The oncologic feasibility of the procedure remains to be determined; however, the currently available interim results indicate that even oncologic outcomes will not be inferior to those of open radical distal pancreatosplenectomy.

Key words: Pancreatic cancer; Laparoscopic pancreatectomy, Robotic pancreatectomy

Core tip: Minimally invasive (laparoscopic or robotic) radical distal pancreatosplenectomy is technically feasible and safe for margin-negative resection in well-selected left sided pancreatic cancer. Generally acceptable potential indications are proposed to include the following: (1) pancreas-confined tumors; (2) intact fascia layer between the distal pancreas and left adrenal gland/kidney; and (3) tumor 1-2 cm from celiac axis. The long-term oncologic feasibility remains to be discerned, but the currently available interim results are encouraging. Further clinical experience with this minimally invasive approach for left-sided pancreatic cancer should be accumulated by experienced surgeons. In the near future, surgical approaches should be specified according to the conditions of the individual pancreatic cancer case.

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INTRODUCTION

With recent advancements in laparoscopic experience, techniques, and instruments, laparoscopic surgery has replaced conventional open surgery in most general surgical fields, even in cancer surgery. Despite the potential limitations of conventional laparoscopic surgery, many studies have prov-

en the oncologic feasibility and rationale for laparoscopic surgery in various malignant diseases, such as cancers of the esophagus[1,2], stomach[3,4], liver[5], colon[6,7], *etc*. However, it remains controversial whether minimally invasive surgery should be applied to treat pancreatic cancer.

Pancreatic cancer is known to be one of the most lethal gastrointestinal malignancies. As a monotherapy, margin-negative pancreatectomy can provide the essential clinical conditions for cure, but the resection rate is very low due to the advanced cancer stages that are usually present at the initial diagnosis. In addition, surgical techniques for margin-negative radical pancreatectomy are very difficult and complex procedures, even in the conventional open approach. Therefore, many surgeons greatly fear that the risk of incomplete surgery might arise when applying minimally invasive techniques to treat pancreatic cancers. Moreover, the lack of more advanced laparoscopic techniques and the limited amount of clinical evidence are some of the biggest obstacles to the use of laparoscopic approaches in the treatment of pancreatic cancer.

Still, several currently available studies have suggested that patients with pancreatic cancer may have appropriate backgrounds for the use of a minimally invasive approach to treat well-selected left-sided pancreatic cancers. First, unlike laparoscopic pancreaticoduodenectomy, laparoscopic distal pancreatectomy is generally regarded as a safe and effective treatment modality in benign and borderline malignant diseases[8]. Second, even laparoscopic subtotal (or extended) distal pancreatectomy can be feasible and safe[9]. Third, many laparoscopic gastric surgeons have already proven the oncologic safety and feasibility of laparoscopic perigastric lymph node dissection in the treatment of gastric cancer[10]. Fourth, the concept of radical antegrade modular pancreatosplenectomy (RAMPS)[11] is thought to be a reasonable approach for margin-negative and systemic lymph node clearance in left-sided pancreatic cancer. Fifth, the early detection of small and asymptomatic pancreatic cancer is expected to increase in the near future due to frequent routine medical check-ups. Finally, even though the data remain limited, a few encouraging studies have been published on the feasibility of a minimally invasive approach to pancreatic cancer[12-14].

Various types of minimally invasive pancreatectomy are currently feasible; however, in this review, we will address distal pancreatosplenectomy in the treatment of pancreatic cancer because this surgical procedure is popular and generally regarded as safe. Therefore, it is thought that laparoscopic distal pancreatectomy with splenectomy could be the initial step for generalizing the concept of a minimally invasive approach to well-selected pancreatic cancers.

CONCEPT OF RAMPS AS A MINIMALLY INVASIVE (LAPAROSCOPIC OR ROBOTIC) APPROACH

Strasberg *et al*[11,15] presented this modified distal pancreatosplenectomy technique in pancreatic cancer. In this method, dissection proceeds from right to left after an early division of the pancreatic neck on one of the two posterior dissection planes to achieve negative posterior resection margins. The plane of dissection runs posteriorly in the sagittal plane along the superior mesenteric artery and celiac artery to the level of the aorta and then laterally, either anterior or posterior to the adrenal gland, for tangential margin clearance. The accompanying N1 lymph node dissection is based on the established anatomy of lymphatic drainage of the pancreas. The posterior dissection plane can be actively placed for tangential margin clearance. According to the posterior dissection plane of the pancreas, three types of RAMPS can be generally classified (Figure 1). Compared to the usual conventional technique for distal pancreatosplenectomy (dissection from left to right first and vascular control later[16]), RAMPS is thought to be more in line with general oncologic concepts, such as early vascular control and no-touch isolation with en bloc surgical resection. Therefore, when applying minimally invasive approaches to left-sided pancreatic cancer, the principles behind RAMPS should be incorporated, although the generally acceptable extent to which minimally invasive RMAPS can be applied must be determined first.

DETERMINING THE EXTENT OF MINIMALLY INVASIVE RAMPS AND POTENTIAL INDICATIONS

According to our surgical experiences with left-side pancreatic cancer, bloodless and margin-negative resection is an important factor in treating left-sided pancreatic cancer[14]; other reports have also supported this finding[17,18]. However, the use of combined adjacent organ resection has been associated with large amounts of intraoperative bleeding, transfusion, morbidity, and increased risks of a positive resection margin[19,20].

When correlating between the RAMPS surgical mode and the potential tumor behavior, several relationships can be identified (Figure 2, solid line). For example, in the case where posterior RAMPS 2 is selected for margin-negative resection, as opposed to anterior RAMPS, there is a high probability of a large tumor size, combined resection of adjacent organs, large amounts of intraoperative bleeding, and perioperative transfusions, as well as technically demanding, more aggressive tumor behaviors, such as actual margin positivity, peritoneal seeding, or hidden distant metastasis. In contrast, when considering the current technical feasibility of minimally invasive distal pancreatosplenectomy for bloodless and margin-negative resections, minimally invasive anterior RAMPS is well accepted; however, it would be very technically difficult to obtain margin-negative and bloodless resections in the case of minimally invasive posterior RAMPS 1 or RAMPS 2 (Figure 2, dotted line). Certainly, minimally invasive posterior RAMPS 1 and RAMPS 2 are also feasible [Figure 2, areas (B) and (C)], but it is thought that only a few expert laparoscopic surgeons can be fully responsible for those demanding surgical procedures[21]. Therefore, it is generally recommended that open aggressive pancreatectomy only

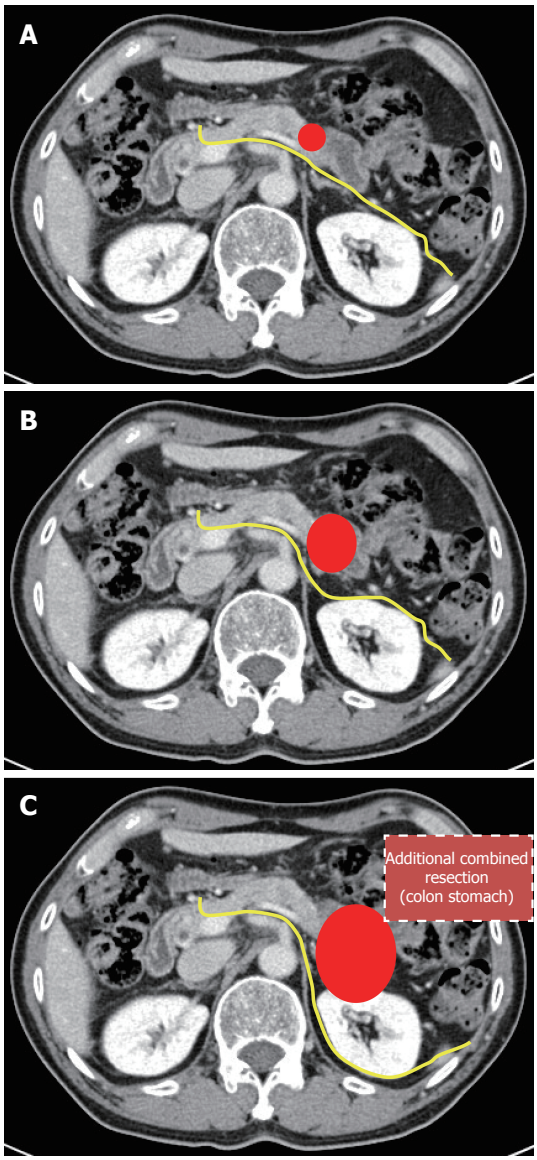

Figure 1 Mode of radical antegrade modular pancreatosplenectomy. A: Anterior radical antegrade modular pancreatosplenectomy (RAMPS); B: Posterior RAMPS 1; C: Posterior RAMPS 2. Dissection plane (yellow line) should be changed for clear tangential margin according to tumor condition (red circle).

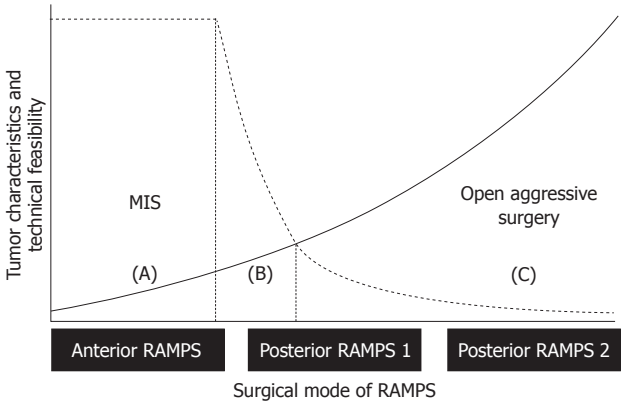

Figure 2 Determining the extent of minimally invasive radical antegrade modular pancreatosplenectomy. The dotted line shows the technical feasibility of bloodless and margin-negative radical antegrade modular pancreatosplenectomy (RAMPS) by a minimally invasive approach, and the solid line represents the biological aggressiveness of tumors, according to the appropriate mode of RAMPS for margin-negative resection. Tentatively, minimally invasive anterior RAMPS is thought to represent a generally acceptable surgical extent for bloodless and margin-negative resections. Oncologically safe posterior RAMPS 1 and 2 might be difficult to perform using a minimally invasive approach. Note the marginal zone of (B). Only a few expert laparoscopic surgeons can be fully responsible for this region. Future directions include widening the area of (B) by means of technical evolution (shifting of the dotted line to the left) and improving early tumor detection (attenuating the slope of solid line). MIS: Minimally invasive surgery.

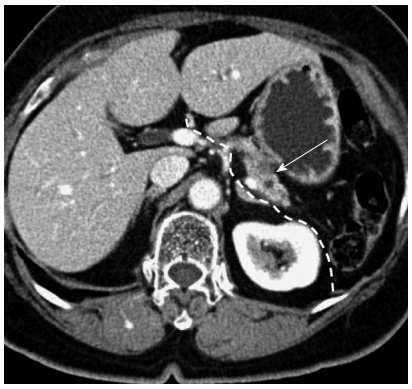

Figure 3 Potential indication for minimally invasive anterior radical antegrade modular pancreatosplenectomy. A 76-year-old female. A relatively pancreas-confined low density mass lesion is noted (arrow). The dotted white line indicates the dissection plane for minimally invasive anterior radical antegrade modular pancreatosplenectomy (RAMPS). The intact fascia layer between the pancreas and left adrenal gland/kidney can facilitate posterior margin clearance when removing the surgical specimen. The tumor is separated from the origin of the splenic artery, necessary for safe vascular control by introducing a minimally invasive technique. The patient underwent laparoscopic anterior RAMPS and has been followed for more than 1 year without evidence of tumor recurrence.

be performed for patients requiring posterior RAMPS 1 and 2. Consequently, when generalizing the concept of minimally invasive approaches to left-sided pancreatic cancer, it would be wise to limit the procedure to anterior RMAPS alone [Figure 2, area (A)][22]. This surgical extent will cover following potential tumor conditions: (1) pancreas-confined tumors; (2) intact fascia layer between the distal pancreas and left adrenal gland/kidney; and (3) tumor 1-2 cm from celiac axis (Figures 3 and 4).

CURRENT CLINICAL PRACTICE OF THE MINIMALLY INVASIVE APPROACH TO LEFT-SIDED PANCREATIC CANCER

Primitive evidence

Until now, many studies have proven the clinical benefit of laparoscopic distal pancreatectomy, with or without splenectomy, in benign and borderline malignant pancreatic disease. However, only a few previous studies have reported the laparoscopic approach for left-sided pancreatic cancer with available long-term survival outcomes[12,23-27]. Since Gagner *et al*[28] first reported laparoscopic distal pancreatectomy, with the advance of laparoscopic techniques and experiences, several other studies have been published, showing the technical feasibility,

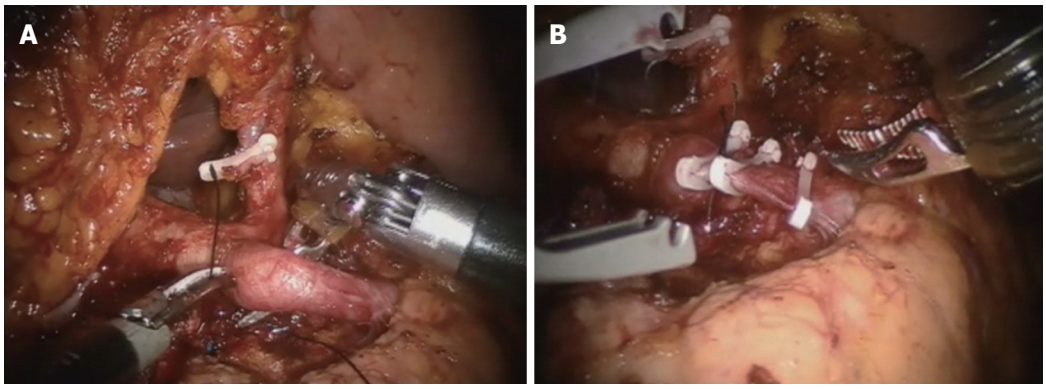

Figure 4 Adequate distance between celiac axis and tumor. Robotic anterior radical antegrade modular pancreatosplenectomy. The origin of the splenic artery is isolated (A) and ligated (B) by the robotic surgical system. For technically and oncologically safe minimally invasive vascular control, some cancer-free space is extremely necessary.

safety, and clinical benefit of laparoscopic distal pancreatectomy over open distal pancreatectomy. However, most reported cases of pancreatic cancer (ductal adenocarcinoma) treated by laparoscopic distal pancreatectomy were incidentally included in those series. As a result, we cannot fully assess the surgical quality based on relevant oncologic concepts. In addition, the lack of information on tumor characteristics, such as pT stage, pN stage, number of retrieved lymph nodes, margin status, and survival outcomes, creates difficulties in determining the oncologic feasibility of the laparoscopic approach to the left-sided pancreatic cancer[24-27,29,30]. For example, in one collective review performed in 2009[31], a final diagnosis of pancreatic ductal adenocarcinoma was found in 51 patients (9.8%, 51 out of 588 patients). However, the margin status was available in only 20 patients (39%). In addition, the number of retrieved lymph nodes in patients with pancreatic cancer was reported in only three articles[26,32,33] (12.5%, 3 of 24 articles identified). Not surprisingly, there is still a lack of long-term survival outcomes. Despite the efforts of several surgeons to perform laparoscopic distal pancreatectomy for pancreatic cancer, it was found that there is a substantial lack of evidence on the oncologic outcomes and surgical quality. Consequently, for the last several decades, we were uncertain whether the minimally invasive approach to left-sided pancreatic cancer was appropriate.

Intermediate evidence

Recently, several studies have been published that focused on the question of whether laparoscopic distal pancreatectomy is oncologically feasible.

DiNorcia *et al*[34] reported their experiences with laparoscopic distal pancreatectomy between 1991 and 2009. Seventy-one patients underwent laparoscopic distal pancreatectomy, and only 9 patients (12.7%) were reported to have malignant pathologies, including 3 cases of pancreatic ductal adenocarcinoma. Long-term survival outcomes were not analyzed; however, the margin-negative resection rate (2.8% *vs* 13%, $P < 0.01$) and mean number of retrieved lymph nodes [6 (range: 2.5-12.0) *vs* 8 (range: 3.0-13.0), $P = 0.29$] were shown to be comparable with those in a conventional open approach.

A recent multicenter analysis reported by Kooby *et al*[13] has provided the most encouraging and impressive evidence, considering the lack of long-term oncologic evidence of laparoscopic approaches to left-sided pancreatic cancer. This study showed that laparoscopic distal pancreatectomy is able to provide similar short- and long-term oncologic outcomes to those obtained with open distal pancreatectomy and suggested that laparoscopic distal pancreatectomy is an acceptable approach for the resection of the left-sided pancreatic cancer in selected patients. In the matched analysis of the overall survival for the patients undergoing an open ($n = 70$) versus a laparoscopic distal pancreatectomy ($n = 23$) for pancreatic cancer, the median survival was comparable among the two the groups (median 16 mo, $P = 0.71$).

In addition, Kim *et al*[35] also published the long-term outcomes of patients who were postoperatively diagnosed with malignancies after laparoscopic distal pancreatectomy. Of the 88 patients who underwent a laparoscopic distal pancreatectomy, 11 (12.5%) were subsequently diagnosed with malignancies in their postoperative pathologic reports. Pancreatic ductal adenocarcinoma was the most common (5 out of 11 patients), followed by invasive intraductal papillary mucinous neoplasm ($n = 3$), neuroendocrine carcinoma ($n = 1$), and so forth. During the follow-up period (range, 3-60 mo), they reported only 1 patient who died of cancer; all others were still alive. Thus, the authors carefully concluded that the postoperative outcomes among patients who were diagnosed postoperatively with malignant pancreatic disease are acceptable.

Although these retrospective studies were not able to suggest either standardized surgical procedures or proper indications, they did suggest potential oncologic outcomes and verify the technical feasibility of the laparoscopic approach to left-sided pancreatic cancer.

Recent advance evidence

More encouraging clinical data with intent-to-treat for

Table 1 Recent studies on minimally invasive radical antegrade modular pancreatosplenectomy and open distal pancreatectomy in left-sided pancreatic cancer *n* (%)

Characterisc	Minimally invasive distal pancreatosplenectomy					Open distal pancreatosplenectomy				
	Fernández-Cruz *et al*[12]	Song *et al*[37]	Choi *et al*[36,38]	Magge *et al*[40]	Marangos *et al*[41]	Kanda *et al*[48]	Kooby *et al*[23]	Kang *et al*[14]	Mitchem *et al*[22]	Okada *et al*[50]
Surgical technique	RAMPS-based	RAMPS- based	RAMPS- based	RAMPS-based	Conventional technique-based	NA	NA	Conventional technique-based	RAMPS-based	Standard DP
Indication	Anatomic dissection No combined resection	No distant metastasis Not locally advanced	Relatively pancreas-confined, intact fascia layer, apart from celiac axis	Allegedly the same as open surgery, even when allowing adjacent organ combined resection	Apparently the same as open surgery, even when allowing adjacent organ combined resection	Resectable, no distal metastasis, no peritoneal seeding, no major vascular invasion	NA	Resectable, no distal metastasis, no peritoneal seeding, no major vascular invasion	Resectable, no distant metastasis, no peritoneal seeding	Resectable, not invading major vessel
Patients (*n*)	10	24	9	28	21	51	70	27	47	36
Age (yr)	NA	NA	64.8 (54-76)	67 (60-75)	63.1 (49-83)	62.7 (38-79)	65.9 ± 11.1	60.5 (47-75)	64.5 ± 10.3	68
Gender (male/female)	NA	16/8	5/4	9/19	6/15	34/17	27/43	20/7	20/27	23/13
Tumor size (cm)	NA	2.6 (1.4-10)	2.5 (1.2-6)	3.0 (2.2-3.6)	4.8 (1.0-10)	7 (< 2 cm) 44 (> 2 cm)	3.5 ± 1.4	3.9 (2.5-5.3)	4.4 ± 2.1	NA
pT stage (T1/T2/T3/T4)	NA	NA	0/0/9/0	NA	NA	2/5/20/24/0[1]	NA	1/4/20/2	1/4/41/1	1/2/15/17/0/1/[1]
Retrieved LNs	14.5 (6-20)	10.3 ± 8.6	10.8 (2-23)	11 (8-20)	7.4(0-26)	NA	12.3 ± 8.3	11.3 (5-18)	18.0 ± 11.7	NA
pN stage (N0/N1)	5/5	NA	2/7	12/16	14/9	25/26	NA	15/12	26/21	19/17
R0(%)/R1/R2	9 (90)/1/0	22 (91.7)/2/0	9 (100)/0/0	24 (86)/4 (14)/0	19 (90.5)/2/0	38 (74.5)/13	46/24	3/4/2020	81%/19%/0%	29 (80.5%)/6/1
Operation time (min)	320 (280-330)	225 (95-360)	368.3 (180-700)	260 (220-340)	NA	NA	216 ± 69	274 (97-453)	243.6 ± 93.5	203 (128-276)
Blood loss	720 (300-1300)	NA	360.1 (trace-1350)	200 (150-300)	NA	NA	751 ± 853	643.3 (100-1200)	744.3 ± 570.4	700 (10-2850)
Hospital stay (d)	8 (7-11)	9.5 (5-22)	9 (4-16)	6 (4-6)	5	NA	9.4 ± 4.7	21.2 (7-24)	11.3 ± 6.8	NA
Adjuvant chemotherapy	10 (100)	17 (70.8)	9 (100)	17 (100)	NA	NA	45 (64)	NA	NA	NA
Follow-up period (mo)	NA	9.95 (1.3-48.5)	20.7 (5.1-45.9)	NA	21.1 (0.5-108)	17.1	10	NA	26.4	25
Oncologic outcome	Median survival 14 mo	2-yr overall survival, 85.2%	2-yr disease free survival, 83.3%	NA	Median survival 19 mo	1, 3, 5-yr overall survival rate, 57.2%, 12.4%, 6.2%	Overall survival 16 mo	Median survival 27.9 mo, 5-yr survival, 28.9%	Median survival 25.9 mo, 5-yr survival, 30.4%	Median survival 32 mo, 2-yr survival 52%

Data are confined to pancreatic ductal adenocarcinoma alone. [1]UICC Cancer Staging System. NA: Not available; RAMPS: Radical antegrade modular pancreatosplenectomy.

minimally invasive approach to left-sided pancreatic cancer has been published. When considering RAMPS-based laparoscopic approaches to treating left-sided pancreatic cancer, only 5 studies investigated the interim or long-term follow up results on the safety and feasibility of minimally invasive distal pancreatectomy for pancreatic cancer (Table 1). A group in Barcelona[12] reported that the median survival of pancreatic cancers treated by laparoscopic RAMPS was 14 mo, comparable to the usual pancreatic cancers that were treated with an open surgery. This report is thought to be first to reveal the technical and oncologic feasibility of laparoscopic RAMPS in left-sided pancreatic cancer. In addition, despite the limited follow-up period, current interim results[36-38] strongly suggest that the possible oncologic outcomes following minimally invasive RAMPS will not be inferior to those of conventional open surgery in well-selected left-sided pancreatic cancer.

A group from Pittsburgh recently published their institutional historical experiences of minimally invasive (laparoscopic and robotic) distal pancreatectomy[39]. A total of 27 patients were reported to have undergone minimally invasive distal pancreatectomy for the treatment of pancreatic ductal adenocarcinoma. When excluding conversion cases

during laparoscopic distal pancreatectomy, the margin-positive resection rate was reported to be 4%, and the capacity for lymph node retrieval was up to 17 (range 10-19); these results are comparable with those of robotic distal pancreatectomy [R1 resection rate, 0% and nodal harvested, median 19 (range 17-27)], suggesting an acceptable quality of surgery in treating pancreatic cancer. They also analyzed retrospective 62 consecutive patients undergoing open distal pancreatectomy (ODP = 34) and minimally invasive distal pancreatectomy (MIDP = 28 with 5 conversions) for pancreatic ductal adenocarcinoma[40]. It was shown that overall survival after ODP or intended MIDP was similar after adjusting for comorbidity and year of surgery [relative hazard, 1.11 (95%CI: 0.47-2.62)]. These two studies still lack long-term oncologic outcomes (median follow up of 21 mo), however, no evidence was detected that MIDP was inferior to ODP in treating pancreatic cancer.

On the other hand, Marangos *et al*[41] published an interesting paper about their surgical experiences with laparoscopic distal pancreatosplenectomy for pancreatic exocrine carcinoma. Since 1997, they reported removing all lesions in the body and tail of the pancreas laparoscopically, and 29 patients with pancreatic cancer (11.6%, 29 out of 250 patients) underwent laparoscopic distal pancreatosplenectomy. Their approach was not based on RAMPS but rather on the conventional left-to-right technique. In addition, they did not perform formal lymph node dissection; instead, they only removed the enlarged or suspicious regional lymph nodes. The dissection plane and resection margins were carefully guided by laparoscopic intraoperative ultrasound. They reported an overall 93% R0 resection rate with a median survival of 23 mo (in particular, 19 mo for 21 pancreatic ductal adenocarcinomas), which is also comparable to the best open series[15,42]. It was noted that the median number of retrieved lymph nodes was smaller (5 nodes), but this did not translate into poor oncologic outcomes, again reminding us of the outcomes of previous prospective randomized controlled studies on standard and extended pancreaticoduodenectomy in the treatment of pancreatic head cancer[43-45]. In addition, in comparison with the oncologic outcomes from open radical surgery, perioperative and oncologic outcomes appear to be comparable between the minimally invasive radical distal pancreatectomy and the open approach (Table 1). One of the most significant weak points of the minimally invasive approach to pancreatic cancer is that the oncologic outcomes are still based on a short-term follow-up period, compared to that of open radical pancreatectomy[14,15,46-48]. However, recently, the single-center-based Pittsburgh group[40] reported a comparative analysis, including long-term survival, of 34 patients with open radical pancreatectomy and 34 with minimally invasive distal pancreatectomy in pancreatic ductal adenocarcinoma to determine the oncological safety and efficacy of minimally invasive surgery. They demonstrated no significant difference between two groups in tumor size (3.0 cm *vs* 3.0 cm), radiologic stage (ⅠA/ⅠB/ⅡA/ⅡB, 3/12/10/6 *vs* 3/11/5/4), margin-negative resection (88% *vs* 86%), power of lymph node retrieval (12 *vs* 11), or lymph node metastasis (38% *vs* 57%) and similar postoperative complications, leading to equivalent survival in propensity score-adjusted overall survival analysis [relative hazard, 1.11 (95%CI: 0.47-2.62), $P = 0.80$]. Along with the multicenter case-matched analysis by Kooby *et al*[13], this study provides powerful evidence to support the technical feasibility of minimally invasive radical oncologic surgery. The study further shows that the quality of surgical specimens is quite acceptable and provides encouraging oncologic survival outcomes.

CHALLENGING ISSUES

Combined and vascular resection

Distal pancreatectomy with *en bloc* celiac axis resection (DPCAR) has been introduced for locally advanced left-sided pancreatic cancer involving the common hepatic artery and/or celiac axis, with perineural invasion in the nerve plexus surrounding these arteries[49,50]. In particular, Okada *et al*[50] recently concluded that DP-CAR is feasible and should be reserved for patients without tumors infiltrating either the portal venous or arterial system. Considering these circumstances, DP-CAR is suggested to be a safe procedure, similar to standard distal pancreatectomy in well-selected patients. Recent technological innovations and extensive surgical experiences are expanding the clinical applications for laparoscopic distal pancreatectomy. As a result, the technical feasibility of minimally invasive distal pancreatectomy with combined celiac trunk or portal vein resection has also been reported. Cho *et al*[21] reported the technical feasibility of pure laparoscopic DP-CAR, finding it safe and feasible to achieve R0 resections in selected patients with locally advanced pancreatic cancer. Giulianotti *et al*[51] and Boggi *et al*[52] also reported robotic pancreatectomy with vascular resection for locally advanced pancreatic tumors. In addition, Kendrick *et al*[53] reported 11 patients who underwent total laparoscopic pancreaticoduodenectomy with major venous vascular resection, including laparoscopic end-to-end vascular reconstruction, patch, and renal vein graft. However, patients with left-sided pancreatic cancer invading isolated superior mesenteric vein-splenic vein-portal vein confluence are rare, as most cases of pancreatic cancer are usually associated with celiac axis and superior mesenteric artery invasion[54], which will be determined as locally invasive pancreatic cancer (unresectable). In general, pancreatic surgeons must consider possible combined vascular resection in their surgical approaches to pancreatic cancer and should be prepared to meet this surgical demand. However, how many surgeons can be responsible for this advanced laparoscopic technique? How should the educational system be modified to reproduce this surgical skill?

Is only RAMPS the ideal approach?

The surgical approach of RAMPS has demonstrated favorable oncologic outcomes in treating left-sided pancreatic cancer[11,15,22,55]. The basic concept of RAMPS is,

of course, oncologically sound and reasonable; however, it is notable that no randomized controlled studies have tested the oncologic superiority between RAMPS and conventional radical distal pancreatectomy. There are several comparable reports showing similar survival outcomes to RAMPS[14,41,56]. An RCT should be performed to test whether the RAMPS procedure is superior to standard distal pancreatectomy. However, it is very difficult to organize a successful trial. Mitchem *et al*[22] have already commented on this issue, as follows: "However, the disparity between the number of cases available for study and the number required for a randomized trial makes this goal unattainable".

FUTURE PERSPECTIVES ON MINIMALLY INVASIVE LEFT-SIDED RADICAL PANCREATECTOMY

As shown in other gastrointestinal cancer surgeries, there has been an increasing clinical effort to apply the laparoscopic approach to left-sided pancreatic cancer. However, procedural standardization and surgical indications have not yet been established. Currently, RAMPS seems to be a reasonable approach, with encouraging oncologic outcomes in the treatment of left-sided pancreatic cancer[22]. Nevertheless, it might be difficult to expand the use of minimally invasive RAMPS to all left-sided pancreatic cancers because these cancers are usually found in advanced cancer stages. However, the clinical conditions required to widen the area (B) in Figure 2, such as technical evolution (right sided-shift of dotted line in Figure 2) and the early detection of the cancer (attenuating slope of the solid line), would facilitate the clinical application of minimally invasive RAMPS in well-selected cases of left-sided pancreatic cancer.

Recently, the use of radical pancreatectomy followed by neoadjuvant chemoradiation therapy has been successfully applied for the treatment of advanced pancreatic cancers[57-59]. In considering the future circumstances of potent chemoradiation therapy for the treatment of pancreatic cancers, minimally invasive RAMPS following neoadjuvant chemoradiation therapy would be another potential option for well-selected patients. In particular, considering the technical advances of combined vascular resection in treating pancreatic cancer, the indications for minimally invasive radical distal pancreatectomy should be expanded in the near future. In addition, many academic institutions seem to be carefully accumulating clinical experience with the minimally invasive resection of left-sided pancreatic cancer. Perhaps in the near future, more relevant clinical evidence with adequate long-term follow-up and qualified oncologic outcomes will become available, leading to the oncologic feasibility of minimally invasive left-sided pancreatectomy in pancreatic cancers. Generally, these conclusions will be influenced by selection bias from the retrospective nature of studies. However, these identified instances of selection bias, in turn, will become potential selection criteria for minimally invasive radical pancreatectomy in distal pancreatic cancers, especially given the difficulty of establishing an RCT in the present circumstances.

CONCLUSION

More than 20 years have passed since the first laparoscopic cholecystectomy was performed in the late 1980s. Tremendous improvements in the surgical techniques, experiences, and new effective instruments have successfully expanded the indications for laparoscopic surgery. Minimally invasive (laparoscopic and robotic) radical pancreatectomy in well-selected left-sided pancreatic cancers is feasible under general oncologic concepts; however, solid clinical evidence is still lacking. Further clinical experience with a minimally invasive approach to left-side pancreatic cancer must be carefully accumulated by experienced surgeons. The oncological feasibility should be addressed in greater detail based on long-term survival outcomes. However, we should not overlook that the currently available interim results demonstrating minimally invasive left-sided radical pancreatectomy are not inferior to those of conventional open radical pancreatectomy.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Optimum chemotherapy in the management of metastatic pancreatic cancer

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Abstract

Pancreatic cancer is one of the most devastating solid tumors, and it remains one of the most difficult to treat. The treatment of metastatic pancreatic cancer (MPC) is systemic, based on chemotherapy or best supportive care, depending on the performance status of the patient. Two chemotherapeutical regimens have produced substantial benefits in the treatment of MPC: gemcitabine in 1997; and FOLFIRIONOX in 2011. FOLFIRINOX improved the natural history of MPC, with overall survival (OS) of 11.1 mo. *Nab*-paclitaxel associated with gemcitabine is a newly approved regimen for MPC, with a median OS of 8.6 mo. Despite multiple trials, this targeted therapy was not efficient in the treatment of MPC. Many new molecules targeting the proliferation and survival pathways, immune response, oncofetal signaling and the epigenetic changes are currently undergoing phase Ⅰ and Ⅱ trials for the treatment of MPC, with many promising results.

Key words: Metastatic pancreatic cancer; Chemotherapy; Evolution; Recent therapies; Targeted therapy

Core tip: This paper will be the newest study with the most recent updates in the treatment of metastatic pancreatic cancer. After a brief review of the different treatments for metastatic pancreatic cancer, the current treatment options are discussed, as well as novel therapies and approaches in the future.

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INTRODUCTION

Pancreatic cancer (PC) is one of the most aggressive and devastating solid tumors with the worst mortality. The median overall survival (OS) is less than 6 mo, and less than five percent of patients will survive more than 5 years (2% in cases of metastatic pancreatic cancer). The large majority of pancreatic cancers are locally advanced (50%) or metastatic (40%) because of their late diagnoses[1].

PC remains one of the most difficult cancers to treat due to its intrinsic resistance to conventional treatments. Many regimens have been implicated in the treatment of metastatic pancreatic cancers (MPC), but only two have had significant impact: GEMCITABINE, introduced in 1997[2]; and FOLFIRINOX, introduced in 2011[3]. In the era of targeted therapy, the treatment of pancreatic cancer remains based mainly on chemotherapeutical regimens.

Table 1 Summary of the results of four trials associating Gemcitabine and targeted therapies

Ref.	Regimen	ORR	Median PFS (mo)	Median OS (mo)
Philip *et al*[10]	Gem/cetuximab	12.5%	3.4	6.3
	Gem	14.0%	3	5.9
Kindler *et al*[11]	Gem/bevacizumab	13.0%	3.8	5.8
	Gem	10.0%	2.9	5.9
Moore *et al*[12]	Gem/erlotinib	8.6%	3.75	6.24
	Gem	8.0%	3.55	5.91
Rougier *et al*[13]	Gem/aflibercept	ND	3.7	6.5
	Gem	ND	3.7	7.8

ORR: Objective response rate; PFS: Progression free survival; OS: Overall survival; ND: Not determined.

EVOLUTION OF TREATMENT MODALITIES

The primary goals of treatment in MPC are better quality of life, palliation and improved survival. The vast majority of chemotherapeutic drugs have been tried in the treatment of MPC, but few have been selected as standards of care.

Before the approval of GEMCITABINE, 5-FU was the most evaluated agent for MPC, without any survival amelioration. In 1997, Gemcitabine was approved by the FDA, based on the results of a randomized trial, in which Gemcitabine was compared to 5-FU in previously untreated patients. A total of 23.8% of Gemcitabine-treated patients experienced a clinical response, compared with 4.8% of 5-FU-treated patients (P = 0.0022), while the median survival was only extended by 1.24 mo (5.65 *vs* 4.41) in favor of patients receiving Gemcitabine (P = 0.025). The one-year survival rate was 18% for Gemcitabine patients and 2% for 5-FU patients[2].

Since the Gemcitabine era, many gemcitabine-based combination therapies have been widely evaluated over the past decade. Most trials have used a second cytotoxic agent, such as 5-FU[4], capecitabine[5], oxaliplatin[6], cisplatin[7], irinotecan[8] and pemetrexed[9], or a targeted therapy, such as cetuximab[10], bevacizumab[11], erlotinib[12] and aflibercept[13], administered in combination with gemcitabine (Table 1). However, despite a modest improvement in progression-free survival in some trials, a significant benefit in overall survival could not be demonstrated for the majority of these combination therapies.

Of all of these treatments, eroltinib, which positively impacted overall survival, was approved for the treatment of metastatic pancreatic cancer[10]; the addition of bevacizumab to gemcitabine-erlotinib did not lead to a statistically significant improvement in OS[14]. A trend toward better survival was also observed with a gemcitabine-capecitabine regimen. Finally, two meta-analyses, the first by Heinemann *et al*[15] and the second by Sultana *et al*[16], concluded that there was a significant survival benefit when gemcitabine was associated with another agent (platinum and 5-FU derivatives) in patients with good performance status. A recent retrospective study by Khalil *et al*[17] in 2013 reported that adding erlotinib to gemcitabine-cisplatin did not appear to improve OS in MPC.

In 2007, we reported on a phase Ⅱ clinical trial assessing a gemcitabine-free regimen based on FOLFOX 6, with promising results. A partial response was observed in 27.5% of the patients and stable disease in 34.5%[18]. Our study and the study by Louvet *et al*[6], which associated gemcitabine and oxaliplatine (RR of 26.8%, the highest with any gemcitabine-based regimen), highlighted the potential role of oxaliplatine in the treatment of MPC.

A second revolution marked the history of MPC in 2011, when Conroy *et al*[3] reported for the first time in NEJM a significant improvement in OS using a gemcitabine–free regimen-the FOLFIRIONOX regimen, based on three chemotherapeutic drugs: 5-FU, irinotecan and oxaliplatine. In this study, the median OS of the patients receiving FOLFIRINOX was 11.1 mo compared to 6.8 mo in the group of patients receiving gemcitabine alone, with an objective response rate of 31.6% compared to 9.4% in favor of the FOLFIRINOX arm. However, more adverse events, such as febrile neutropenia, thrombocytopenia, sensory neuropathy and diarrhea, were noted in the group of patients receiving FOLFIRINOX. This regimen was considered an option for the treatment of patients with MPC and good performance status[3]. A recent study demonstrated that FOLFIRINOX significantly reduced quality of life impairment compared with gemcitabine in patients with MPC[19].

Since the results with the FOLFIRINOX gemcitabine-free regimen, a new attempt with gemcitabine-based combination therapy revealed promising results. Another agent added to gemcitabine was the *nab*-paclitaxel, an albumin-bound nanoparticle form of paclitaxel that increases the tumor accumulation of paclitaxel through binding of albumin to SPARC. A randomized phase Ⅲ study that compared a combination of *nab*-paclitaxel and Gemcitabine weekly to gemcitabine alone showed a significant improvement in overall survival of 8.5 mo *vs* 6.7 mo (P < 0.05) and a response rate of 23% *vs* 7%[20]. An important prognostic biomarker in patients with MPC receiving *nab*-paclitaxel is SPARC; a positive SPARC status in these patients was associated with a significant increase in OS[21].

CURRENT TREATMENT OPTIONS

Treatment is systemic, based on chemotherapy or best supportive care, depending on the performance status of the patient.

In patients with limited performance status, Gemcitabine as monotherapy is the uniquely approved treatment; another alternative is best supportive care. In patients with good performance status, many chemotherapeutical regimens are available (Table 2). Gemcitabine is still considered a possible option[1]. FOLFIRINOX offers the best overall survival and response rate in MPC, but it causes many side effects. Gemcitabine associated with *nab*-paclitaxel offers the second best overall survival, with fewer side effects compared to FOLFIRINOX[16,18].

Table 2 The approved chemotherapeutical regimens for metastatic pancreatic cancer in patients with good performance status

Ref.	Regimen	ORR	Median OS (mo)	Median PFS (mo)
Burris *et al*[2]	Gemcitabine	ND	5.65	2.33
	5-FU	ND	4.41	0.92
Conroy *et al*[3]	FOLFIRINOX	31.6%	11.1	6.4
	Gemcitabine	9.4%	6.8	3.3
Moore *et al*[12]	Gemcitabine/ erlotinib	8.6%	6.24	3.75
	Gemcitabine	8.0%	5.91	3.55
Daniel *et al*[20]	Gemcitabine/ *nab*-paclitaxel	23%	8.5	5.5
	Gemcitabine	7%	6.7	3.7

ORR: Objective response rate; OS: Overall survival; PFS: Progression free survival; ND: Not determined.

Table 3 The adverse events of three approved regimen for metastatic pancreatic cancer reported in NEJM 2011 and ASCO 2013

Adverse events	Gemcitabine	FOLFIRINOX	Gemcitabine/ *nab*-paclitaxel
Neutropenia	21%	45.7%	38%
Febrile neutropenia	1.2%	5.4%	3%
Thrombocytopenia	3.6%	9.1%	13%
Fatigue	17.8%	23.6%	17%
Diarrhea	1.8%	12.7%	6%
Peripheral neuropathy	0%	9.0%	17%

A comparison between the side effects of these three regimens is resumed in the Table 3. Erlotinib remains the unique targeted therapy approved for the treatment of MPC in combination with gemcitabine. Gemcitabine combined with cisplatin or capecitabine can be a reasonable choice in some cases. Patients with MPC and good performance status can also be included in different phase I or Ⅱ clinical trials. All of the approved treatments for MPC in patients having poor and good performance status are reviewed in Figure 1.

The second-line treatment for MPC has been evaluated in only a few trials. The general guidelines for treatment are to use fluoropyrimidine-based chemotherapy if the patient was previously treated with gemcitabine-based chemotherapy and gemcitabine-based chemotherapy if previously treated with fluoropyrimidine-based therapy[22]. A phase Ⅱ trial investigated whether the association of capecitabine with oxaliplatin was active in gemcitabine-pretreated patients with MPC, especially patients with a good performance status and those who responded to first-line chemotherapy[23]. A phase Ⅲ trial comparing the OFF regimen (oxaliplatin; 5-FU; folinic acid) to best supportive care provided first-time evidence for the benefit of second-line chemotherapy in MPC, manifested by prolonged survival time[24]. Palliative radiotherapy has been proposed as salvage therapy for patients with severe pain refractory to narcotics[22].

Novel therapies and approaches

Epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF) have been considered, for the last decade, the two main targets that should be studied in MPC. Many trials have combined gemcitabine with an anti-angiogenic drug or a tyrosine-kinase inhibitor (Table 1); all of these trials have had negative results, except for the combination of gemcitabine and erlotinib, as mentioned above.

After multiple failures with targeted therapy for MPC based on anti-EGFR and anti-VEGF, many new concepts for treating MPC are being elaborated, including the targeting of tyrosine kinase signaling, cascade elements, the stromal reaction, the immune response, oncofetal signaling and epigenetic changes[25].

IFG1R, MEK, PI3K, AKT, and mTOR are actually the most frequent signaling pathway targets evaluated in the treatment of MPC. A phase Ⅱ trial reported that ganitumab (AMG 479), an mAb antagonist of insulin-like growth factor 1 receptor, combined with gemcitabine showed a trend toward improved 6-mo survival and overall survival rates[26]. Many other trials had negative results: selumetinib (AZD6244), a selective MEK inhibitor compared to capecitabine as a second-line treatment after gemcitabine, did not demonstrate any statistically significant difference in overall survival[27]; an oral m-TOR inhibitor (RAD001) had minimal clinical activity in gemcitabine-resistant MPC[28].

Immunotherapy is one of the promising new concepts introduced in the treatment of MPC. A phase I study of an agonist of CD40 monoclonal antibody (CP-870, 893), in combination with gemcitabine, was well tolerated in patients with MPC and was associated with anti-tumor activity[29]. Ipilimumab (anti-CTLA-4), another immunotherapeutic option approved for metastatic melanoma[30], was considered ineffective in the treatment of MPC after the results of a phase Ⅱ trial; association of these agents with other agents could probably have more promising results[31].

Another approach in the treatment of MPC is the targeting of oncofetal signaling, which is responsible for tumor progression and resistance to chemotherapy in PC. One of the most altered pathways incriminated in the development of PC is the Notch pathway[32]; the activation of γ-secretase is the primum movens of activation of Notch signaling. Preclinical data suggested that a selective γ-secretase inhibitor (PF-03084014) had greater anti-tumor activity in combination with gemcitabine in PC, providing a rationale for further investigation of this combination in PC[33]. Many other trials are evaluating agents targeting the stromal reaction and epigenetic changes[34,35].

Another targeted therapy, AGS-1C4D4, a fully human monoclonal antibody against prostate stem cell antigen, was evaluated with gemcitabine in a randomized phase Ⅱ study of untreated MPC, with achievement of its primary end point in demonstrating improved 6-mo SR[36]. All of the recent phase Ⅱ trials studying the new agents in the treatment of MPC are summarized in

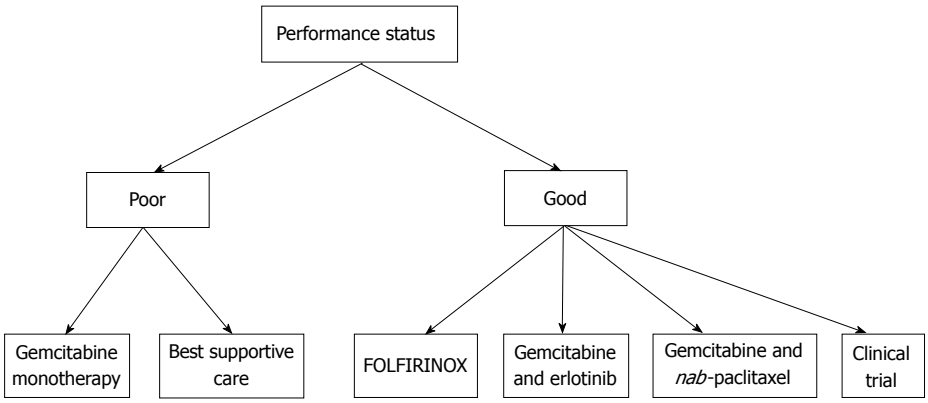

Figure 1 A schema representing the approved treatment for metastatic pancreatic cancer in patients having poor or good performance status.

Table 4 Recent phase Ⅱ trials studying new agents in the metastatic pancreatic cancer

Reference	New agents	Agents target	Phase of the study and targeted population	Arms of the study	Conclusion of the study
Kindler *et al*[26]	Ganitumab (AMG479)	mAb antagonist of insulin-like growth factor 1 receptor	Phase Ⅱ; untreated MPC patients	Gem/ganitumab *vs* gem	Improved 6-mo survival rate and OS
Bodoky *et al*[27]	Selumetinib (AZD6244)	Selective MEK inhibitor	Phase Ⅱ; second line treatment after gemcitabine	Selutimumab *vs* capecitabine	No significant difference in OS
Wolpin *et al*[28]	Everolimus (RAD001)	m-TOR inhibitor	Phase Ⅱ; second line treatment after gemcitabine	Everolimus (single arm study)	Minimal clinical activity
Royal *et al*[31]	Ipilimumab (MDX010)	Anti-CTLA4	Phase Ⅱ; untreated MPC patients	Ipilimumab (single arm study)	Ineffective in the treatment of MPC
Wolpin *et al*[36]	AGS-1C4D4	mAb to prostate stem cell Antigen	Phase Ⅱ; untreated MPC patients	Gemcitabine/AGS-1C4D4 *vs* gemcitabine	Improved 6-mo survival rate

MPC: Metastatic pancreatic cancer; OS: Overall survival.

Table 4.

Many new targets and genes that play roles in the pathogenesis and progression of PC are being evaluated in animals or in cancer cells for their potential diagnostic and therapeutic implications: mucin (myc) was studied by Rachagani *et al*[37], transketolase by Wang *et al*[38] and aberrant CD20 expression by Chang *et al*[39].

The combination of these novel therapies and approaches could positively affect the history of MPC.

CONCLUSION

Despite multiple trials and their major efforts, PC remains resistant to chemotherapy and targeted therapy. It seems that the results obtained with chemotherapy, targeted therapy and their combination in MPC have reached a plateau, with significant, but modest, amelioration of OS of less than one year. Stratified or personalized therapy is totally absent in the treatment of MPC, due to the absence of prognostic or therapeutic markers and the lack of molecular profiling modalities. Many trials are currently being conducted to explore new targets in the tumorigenesis and proliferation of PC. Finally, the combination of these novel therapies with personalized medicine might offer promising results in patients with MPC.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (14): Pancreatic cancer

Screening and early detection of pancreatic cancer in high risk population

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Abstract

Pancreatic cancer is a serious growing health issue in developed countries. For patients diagnosed with pancreatic cancer, the five year survival rate is below 5%. One major important reason leads to the poor survival rate is lack of early detection of pancreatic cancer. Over 80% of the patients are diagnosed in advanced disease stages. Screening for pancreatic cancer is a desirable option for high risk individuals to allow early detection and treatment of curable pancreatic neoplasms at a pre-invasive stage. This article highlights the need, endpoint, population, method, diagnostic yield, and the problems of current screening programs.

Key words: Pancreatic cancer screening; High risk population; Pancreatic neoplasm; Peutz-Jeghers syndrome

Core tip: Screening for pancreatic cancer is a desirable option for high risk individuals to allow early detection and treatment of curable pancreatic neoplasms at a pre-invasive stage. This article highlights the need, endpoint, population, method, diagnostic yield, and the problems of current screening programs.

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INTRODUCTION

Pancreatic cancer is one of the most lethal diseases despite marked improvement in medical and cancer care over the past years. The number of newly diagnosed pancreatic cancer patients has increased significantly in recent years[1]. The most common histological subtype of pancreatic cancer is adenocarcinoma, which comprises 87% of the pancreatic malignancies. Among the pancreatic cancer patients, there were only 15%-20% diagnosed as "resectable" and surgery was the only way to treat the disease. The majority of the pancreatic cancer patients were diagnosed as unresectable and chemotherapy was the standard treatment to control the incurable disease. The prognosis for patients with pancreatic cancer remains poor. The overall survival rate was 5% combining all stages, 20% for patient with localized disease and 1%-2% for those with distant metastasis. In most cases, pancreatic cancer has progressed before clinical manifestation. Many patients initially thought to have localized and resectable cancer succumb to recurrent or metastatic disease. Hence, there is an urgent need to detect small asymptomatic cancers or precursor lesions, which are potentially curable for the most devastating disease.

OPPORTUNITY AND POTENTIAL WINDOW OF SCREENING

A recent study suggested that there may be a large window and good opportunity for detecting pancreatic cancer when the disease is in earliest and most treatable stages[2]. Quantitative analysis of the timing of the genetic evolution of sporadic pancreatic cancer indicated a time span of at least 10 years between the occurrence of cancer-initiating mutations and the formation of parental nonmetastatic founder cell[2]. Indeed, patients with pancreatic tumors diagnosed incidentally had longer median survival[3] than those with tumors discovered after symptoms appeared, suggesting that early detection of small asymptomatic cancers or precursors lesions may improve the outcome. Identification of high risk populations of pancreatic cancer for screening becomes essential. Distinct clinical and genetic features are thought to increase the risk of pancreatic cancers. It has been estimated about 10% of pancreatic cancer has a familial basis. Hereditary pancreatic cancer includes inherited cancer syndromes with a recognized known germline mutation associated with an increased risk of pancreatic cancer and familial pancreatic cancer with two or more cases of pancreatic cancer in their families. Screening pancreatic cancer in these high risk individuals might be recommended for early detection to improve the prognosis of pancreatic cancer. A multidisciplinary international consortium met to discuss pancreatic screening was held recently[4] and some statements regarding to pancreatic cancer screening were made and voted to guide the pancreatic cancer screening.

DEFINITION OF "SUCCESS" OF SCREENING

A very recent effort made by international cancer of the pancreas screening (CAPS) has proposed to define "successful screening" by detection and treatment of T1N0M0 margin negative pancreatic cancer and high grade dysplastic precursor lesions, including pancreatic intraepithelial neoplasia-3 (PanIN-3), intraductal papillary mucinous neoplasm (IPMN) with high grade dysplasia, and mucinous cystic neoplasm (MCN) with high grade dysplasia[4].

WHO TO SCREEN?

Not all populations with risk of pancreatic cancer need to be screened because there is no evidence that screening for pancreatic cancer is effective in reducing mortality and the harms of screening for pancreatic cancer exceed any potential benefits[5]. Clinical risk factors include age, obesity, smoking, diabetes, and non-genetic chronic pancreatitis are associated with pancreatic cancer. However, the specificity of these factors to pancreatic cancer is low. For example, the risk for developing pancreatic cancer increases with age, mostly in individuals at age over 45. Overweight and obese individuals have an increased risk (odds ratio: 1.8 and 1.22 in males and females, respectively) and earlier disease onset[6]. Current cigarette smokers and former smokers who had quit for less than 5 years also have a higher risk of pancreatic cancer than non-smokers (odds ratio: 1.71 and 1.78 for current smokers and recent past smokers, respectively)[7]. Patients with diabetes are at higher risk for pancreatic cancer (odds ratio: 1.76)[8], and new onset of diabetes may be an early indicator of pancreatic cancer[9]. Several studies have indicated that patients with (non-genetic)chronic pancreatitis had a higher incidence of pancreatic cancer over the general population (odds ratio: 2.23)[10-12].

HIGH RISK POPULATIONS

Screening is suggested in high risk populations, including individuals with lifetime risk of pancreatic cancer over 5% or/and increased relative risk over 5 times proposed by CAPS[4]. Table 1 listed the proposed high risk population to screen and their relatively risk and/or lifetime risk of pancreatic cancer.

PEUTZ-JEGHERS SYNDROME

Peutz-Jeghers (PJ) syndrome is an autosomal dominantly inherited syndrome caused germline *STK11* gene mutations with high penetrance[13]. It is characterized by mucocutaneous pigmentation and hamartomatous polyps of the gastrointestinal (GI) tract. Patient with PJ syndrome have a risk of multiple GI and non-GI cancers. The cumulatively lifetime risk of pancreatic cancer is 36%, with a relatively risk (RR) of 132[14,15]. Patients with PJ syndrome whatever with family history of pancreatic cancer are suggested to be candidates for pancreatic cancer screening[4].

FAMILIAL ATYPICAL MULTIPLE MOLE MELANOMA SYNDROME

Familial atypical multiple mole melanoma syndrome is an autosomally dominant disease with variable penetrance caused by *p16/CDKN2A* gene mutation[16]. It is characterized by familial occurrence of multiple benign melanocytic nevi, dysplastic nevi, and melanoma[17]. Familial Atypical Multiple Mole Melanoma (FAMMM) syndrome is associated with extrapancreatic (sarcomas, endometrial, breast and lung cancers) and pancreatic cancers. The risk of developing pancreatic cancer risk is about 13-22 folds[18-20]. *p16* mutation carriers with one or more affected first degree relative (FDR) with pancreatic cancer should be considered for screening[4].

FAMILIAL BREAST AND OVARIAN CANCER

Familial breast and ovarian cancer syndrome is an autosomal dominantly inherited syndrome associated

Table 1 High risk population and the estimated risk for pancreatic cancer

Syndrome	Gene	RR	Lifetime risk
Peutz-Jeghers syndrome	*STK11/LKB1*	132	36% by age 65 yr
Hereditary pancreatitis	*PRSS1*	53	Male: 11% and 49% by age 50 and 75 yr Female: 8% and 55% by age 50 and 75 yr
FAMMM	*p16*	13-22	16% lifetime risk
Familial breast and ovarian	*BRCA1/2*	3-10	5% lifetime risk
HNPCC	*MLH1, MSH6, MSH2, PMS2*	1.5-9	8.6% lifetime risk
Familial pancreatic cancer			
2 FDR	Unknown	6.4	8%-12% lifetime risk
3 FDR	Unknown	32	40% lifetime risk

FDR: First degree relative; HNPCC: Hereditary non-polyposis colorectal cancer; PRSS1: Cationic trypsinogen gene; FAMMM: Familial atypical multiple mole melanoma syndrome.

with germline mutations of *BRCA1* and *BRCA2* genes. Mutation carriers are at high risk for breast, ovarian, GI cancers (bile duct, gallbladder, stomach, pancreas) and prostate cancers[21-24]. BRCA2 carriers are associated with higher risk of pancreatic cancer (3-10 folds) than BRCA1 carriers (2.3-3.6 folds)[24,25]. *BRCA2* mutation carriers with one or more affected FDR with pancreatic cancer and those with two or more affected family members (even without a FDR) should be considered for screening[4].

HEREDITARY NON-POLYPOSIS COLORECTAL CANCER, LYNCH SYNDROME

The lynch syndrome is associated with mismatch repair genes (*MLH1*, *MSH2*, *MSH6* and *PMS2*). Hereditary non-polyposis colorectal cancer (HNPCC) is characterized by early-onset colorectal cancers and extra-colonic cancers including pancreas[26]. The lifetime risk of pancreatic cancer is 3.7% by age of 70, an 8.6-fold increased risk compared to general population[27]. Patients with Lynch syndrome and one affected FDR with PC should be considered for screening[4].

HEREDITARY PANCREATITIS

Hereditary pancreatitis is a rare inherited disorder. It is transmitted as an autosomal dominant disorder with incomplete penetrance[28,29]. Hereditary pancreatitis is associated with a high risk of pancreatic cancer with a lifetime risk about 40%[30]. In those individuals with a paternal inheritance pattern, the cumulative risk is even approaching 75%[30]. This risk of pancreatic cancer is related to the duration of inflammation[31]. Screening of *PRSS1* (Cationic trypsinogen) mutation carriers with longstanding chronic pancreatitis is being performed within established programs[32].

FAMILIAL PANCREATIC CANCER

Familial pancreatic cancer (FPC) describes families with at least two first-degree relatives with confirmed exocrine pancreatic cancer that do not fulfill the criteria of other inherited tumor syndromes. FPC is also used to describe families with exocrine pancreatic cancer in two or three or more relatives of any degree[33,34]. An indicative pattern of an autosomal dominant trait of inheritance has been identified in 58%-80% of FPC families[35-37]. Previous studies have described an increased risk of developing pancreatic cancer in unaffected FDRs that depends on the number of relatives with pancreatic cancer[38]. Studies of the European Registry of Hereditary Pancreatitis and FPC (EUROPAC) and German national case collection for FPC (FaPaCa) described the phenomenon that patients in younger generations develop the disease about 10 years earlier than their affected parents[36,37]. The proportion of patients younger than 50-year-old appeared to be higher (16%) in FPC families compared to the general population[34]. For individuals with two affected first-degree relatives and individuals with three affected first-degree relatives, the relative risk are 32[39]. The risk of pancreatic cancer seemed to be higher among members of FPC kindred with a young age of onset (younger than 50 years of age) compared with kindred with an age of onset older than 50 years of age. The life-time risk rose to 38% for individuals with three affected first-degree relatives, if one of the affected was diagnosed under the age of 50 years[40].

PALB2 gene was identified as a PC susceptibility gene recently[41]. It is a partner and localizer of *BRCA2*. *PALB2* germline mutations have been detected in up to 3% of patients with familial PC[41-43]. The risk of PC among *PALB2* gene mutation carriers is estimated to be similar to that found for *BRCA2* gene mutation carriers. *PALB2* mutation carriers with one or more affected FDR with PC should be screened[4].

WHEN TO SCREEN?

Patients with hereditary pancreatitis has an higher risk of early onset pancreatic cancer. Screening typically begins at age 40 in *PRSS1* mutation carriers[44]. In other high risk populations, there is no consensus as to whether to recommend initiating screening and the end of screening[4].

SCREEN TECHNIQUES

Up to now, there is no ideal single screening method or screening program for detection of early pancreatic cancer. Serum CA19-9 levels is the most commonly used serum marker in pancreatic cancer. However, the sensitivity and specificity of serum CA19-9 as a diagnostic marker are not good for screening pancreatic

Table 2 Reported pancreatic cancer screening programs and diagnostic yield

Study	Screening modalities	Case (*n*)	Study population	Diagnostic yield upon imaging
Rulyak *et al*[46], 2001	EUS	35	FPC	34.3%
Canto *et al*[56], 2004	EUS	38	FPC, PJS	76%
Canto *et al*[55], 2006	EUS	78	FPC, PJS	22%
Poley *et al*[47], 2009	EUS	44	FPC, FAMMM, PJS	23%
Langer *et al*[48], 2009	EUS + MRCP	76	FPC, PCMS	36%
Verna *et al*[49], 2010	EUS and/or MRCP	51	FPC, FAMMM, HNPCC	EUS: 65% MRI: 33%
Ludwig *et al*[50], 2011	MRCP	109	FPC	8.3%
Vasen *et al*[51], 2011	MRCP	79	FAMMM	20%
Canto *et al*[52], 2012	MRCP, EUS, CT	216	FPC, HBOC, PJS	42.6%
Al-Sukhni *et al*[53], 2012	MRCP	262	FPC, FAMMM, PJS, hereditary pancreatitis	32%

EUS: Endoscopic ultrasonography; MRI: Magnetic resonance imaging; CT: Computed tomography; FAMMM: Familial atypical multiple mole melanoma syndrome; FPC: Familial pancreatic cancer; HBOC: Hereditary breast-ovarian cancer; HNPCC: Hereditary nonpolyposis associated colorectal cancer; IPMN: Intraductal papillary mucinous neoplasia; MRCP: Magnetic resonance cholangiopancreatography; PCMS: Pancreatic carcinoma-melanoma syndrome; PJS: Peutz-Jeghers syndrome.

cancer[45]. The most common screening imaging used for the detection of pancreatic cancer are endoscopic ultrasonography (EUS), computed tomography (CT) and magnetic resonance imaging (MRI) with magnetic resonance cholangiopancreatography (MRCP). EUS is an invasive procedure which might detect lesions smaller than 1 cm. However, the major problem of EUS is its operator dependent. CT scanning demonstrated a low sensitivity to detection pancreatic dysplasia. MRI with MRCP is a non-invasive procedure which could detect earlier ad minor changes in pancreatic parenchymal and (main) pancreatic duct compared to CT scan. Table 2 summarized the reported pancreatic cancer screening programs with the reported diagnostic yield. MRI with MRCP and EUS are considered the most accurate tools for pancreatic imaging as promising recommended tool for screening[46-56]. The major weakness of CT is its radiation exposure and the suboptimal detection rate as a routine screening tool for asymptomatic high risk individuals[4]. MRI with MRCP is less invasive and more objective compared to EUS. It still lacks randomized controlled studies to compare EUS and MRI with MRCP in pancreatic cancer screening in high risk individuals. Regarding to the imaging study as a screening tool, over diagnosis is a major problem which might cause over treatment of a benign lesion. The risk of incorrect diagnosis is particularly high for EUS because of it is an operator-dependent examination with only modest interobserver agreement[57]. Abdominal ultrasound and endoscopic retrograde cholangiopancreatography are not recommended for screening, owing to their low diagnostic sensitivity and the risk of pancreatitis, respectively[4].

EMERGING PROBLEMS AND FUTURE PROSPECTIVE

There are still some unresolved problems in pancreatic cancer screening. First of all, the aim of screening is to find the earliest pancreatic cancer (T1N0M0) or high grade precursor lesions in PanIN, IPMN and MCN. In fact, the high grade PanINs are actually microscopic lesions which might cause some tiny or abnormal findings in imaging. Even with fine needle aspiration, the aspirated substance could not represent the worst condition or whole picture what it is. Secondly, we still have no imaging modality or accurate criteria to differentiate benign pancreatic cystic lesions from malignant cystic tumors with dysplasia or malignancy. There are some proposed "high risk stigmata and worrisome features"[58] to help us for picking up true meaningful or suspected malignant pancreatic cystic lesions or IPMN to avoid unnecessary operations or overtreatment. However, there is still no reliable or good method to different the nature of pancreatic cystic lesions. With the advancement and frequent use of abdominal imaging, more and more incidentally found pancreatic lesions and/or IPMNs are disclosed. How to follow up the increasing numbers of patient with optimal programs to avoid under detection of pancreatic cancer and also to avoid overtreatment will be a great challenge for clinician.

CONCLUSION

Screening pancreatic cancer in high risk populations is suggested to enhance the potential early detection of curable early pancreatic cancer. It is a potential way to improve the outcome of pancreatic cancer. Although some consensus are proposed to be followed, there is still lack of ideal screening method and program at the present time. Further study and advancement for improving the sensitivity and specificity of screen methods to achieve the goal of early detection of pancreatic cancer is warranted in the near future.

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ORIGINAL ARTICLE

Sweet food improves chronic stress-induced irritable bowel syndrome-like symptoms in rats

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Abstract

AIM: To investigate whether palatable sweet foods have a beneficial effect on chronic stress-induced colonic motility and inflammatory cytokines.

METHODS: Adult male rats were divided into 3 groups: control (CON, *n* = 5), chronic variable stress with chow (CVS-A, *n* = 6), and chronic variable stress with chow and sweet food (CVS-B, *n* = 6). The rats were fed standard rodent chow as the chow food and/or AIN-76A as the sweet food. A food preference test for AIN-76A was performed in another group of normal rats (*n* = 10) for twelve days. Fecal pellet output (FPO) was measured for 6 wk during water bedding stress in the CVS groups. The weight of the adrenal glands, adrenocorticotropic hormone (ACTH) and corticosterone levels in plasma were measured. The expression levels of transforming growth factor-β, interleukin (IL)-2, and interferon-gamma (IFN-γ) were measured in the distal part of colonic tissues and plasma using Western blot analysis.

RESULTS: In sweet preference test, all rats initially preferred sweet food to chow food. However, the consumption rate of sweet food gradually decreased and reduced to below 50% of total intake eight days after sweet food feeding. Accumulated FPO was higher in the CVS-A group compared with the CVS-B group over time. All stress groups showed significant increases in the adrenal to body weight ratio (CVS-A, 0.14 ± 0.01; CVS-B, 0.14 ± 0.01) compared with the control group (0.12 ± 0.01, $P < 0.05$). The plasma corticosterone and ACTH levels were significantly higher in the CVS-A (537.42 ± 32.95, 44.44 ± 6.54 pg/mL) and CVS-B (655.07 ± 30.82, 65.46 ± 4.44 pg/mL) groups than in the control group (46.96 ± 13.29, 8.51 ± 1.35 pg/mL, $P < 0.05$). Notably, the ratio of corticosterone to ACTH was significantly increased in the CVS-A group only. Rats exposed to CVS displayed significantly increased expression of IL-2 and IFN-γ in the plasma and distal colon compared to the control group, whereas this effect was significantly attenuated in the CVS-B group.

CONCLUSION: These results suggest that concurrent sweet food ingestion during CVS might have an effect on the reduction of stress-induced colonic hyper-motility and pro-inflammatory cytokine production in rats.

Key words: Irritable bowel syndrome; Colon; Stress; Adrenal hormones; Cytokines; Rat

Core tip: Stress has an important role in the pathogenesis of irritable bowel syndrome (IBS), and palatable foods have been used as an ameliorator for psychological stress. Several reports have supported the hypothesis that palatable foods are used for consolation from psychological stress. Thus we hypothesized that hypothalamic-pituitary-adrenal axis function and immune status in the plasma and colon could be altered by chronic stress, and these changes could be attenuated by concurrent ingestion of sweet food. These results imply that reducing the effect of stress appropriately by any means is important for preventing induces gastrointestinal symptoms in a patient with IBS.

Rho SG, Kim YS, Choi SC, Lee MY. Sweet food improves chronic stress-induced irritable bowel syndrome-like symptoms in rats. *World J Gastroenterol* 2014; 20(9): 2365-2373 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i9/2365.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i9.2365

INTRODUCTION

It is generally accepted that psychological stress induces gastrointestinal (GI) symptoms, including dyspepsia, abdominal pain and increased colonic motility[1]. Stress has an important role in the pathogenesis of irritable bowel syndrome (IBS) as a risk, trigger and perpetuating factor[2]. Although the underlying pathogenesis of IBS still remains unclear, dysregulation of the brain-gut-axis[3] and low grade GI inflammation have recently received attention as potential causes[4].

A patient with IBS exhibits over-activation of the hypothalamic-pituitary-adrenal (HPA) axis to corticotropin-releasing factor (CRF) stimulation or visceral stimulation and exhibits increased pro-inflammatory cytokines in the blood[5,6]. There has been growing evidence regarding the association between IBS and low grade inflammation, such as increased expression of immune and mast cells[7,8] cytokine imbalance in the mucosa[9], and elevated circulating levels of the pro-inflammatory cytokine in IBS patients[10]. Concerning the function of cortisol, such as its role in reducing the number of leukocytes[11] and suppressing the release of interferon-gamma (IFN-γ)[12] and interleukin (IL)-2[13], it can be assumed that stress and the associated HPA axis dysfunction act in concert to contribute to the alteration of GI immune function in the pathogenesis of IBS.

Eating behavior is closely related to stress, and some people preferentially consume palatable food such as chocolate or ice cream during stressful situations[14]. Several reports have supported the hypothesis that palatable foods are used for consolation from psychological stress through the stabilization of CRF in the hypothalamus[15-17]. Therefore, it appears that the consumption of sweet food has beneficial effects on stress-induced physiologic dysfunction. However, it has never been reported whether eating sweet food has an effect on the disordered GI motility and immune status in chronically stressed rats.

In the present study, we hypothesized that the HPA axis function and the immune status in the plasma and colon could be altered by chronic stress, and these changes could be attenuated by concurrent ingestion of sweet food. Therefore, the aim of this study was to investigate the effect of the concurrent eating of sweet food on colonic motility, HPA axis status, and the levels of inflammatory cytokines in the plasma and colon in chronic variable stress (CVS) rats.

MATERIALS AND METHODS

Animals and study design

Animal use protocols (No. WKU09-112) were approved by the Institutional Guidelines of the Committee on Animal Research at the Wonkwang University, and all efforts were taken to minimize animal suffering and to reduce the number of animals necessary according to the guideline recommendations. Seventeen male Sprague-Dawley rats purchased from Samtaco Co., Ltd. (Pyeongtaek, South Korea), weighing approximately 270 g each, were housed in individual cages for ten days for acclimatization. Rats were provided ad libitum access to food and water in 12 h/12 h light-dark cycles at 24 ℃.

The animals were randomly divided into 3 groups: no stress with chow food (CON, $n = 5$), CVS with chow food (CVS-A, $n = 6$), and CVS with chow + sweet food (CVS-B, $n = 6$).

Supplied food and preference test

We used commercial standard rodent chow (60% carbohydrate, 20% protein, 4.5% fat, Purina Mills Inc., St. Louis, MO, United States) as the chow food and AIN-76A (66% carbohydrate, 20% protein, 5% fat, Research Diets Inc., New Brunswick, United States) as the sweet food. Not only does AIN-76A contain a higher carbohydrate percentage, but the source of carbohydrate is also sucrose, which is sweeter than glucose, dextrose and lactose. AIN-76A contains more sucrose than any other purified food (AIN-93G, AIN-93M) or cereal-based chow food. Before the stress experiment, a food preference test for AIN-76A was performed in another group of normal rats ($n = 10$) for twelve days. To diminish neophobia to the novel foods, CVS-B rats were exposed to AIN-76A in advance for 3 d before the experiment.

CVS protocol

The CVS protocol used in this study was modified from the previous study[18]. This protocol was demonstrated to induce visceral hypersensitivity and is suggested as an IBS animal model[18]. The weekly protocol consisted of placement in a small cage (confinement), water bedding, exposure to white noise, a stroboscope, light at night (illumination), and irregular vibration (each rat cage was

Table 1 Experimental schedule for chronic variable stress

Schedule	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
09:00-12:00	Water bedding (1 h)	Vibration (3 h)	Water bedding (1 h)	Confinement (2 h)	Water bedding (1 h)	Vibration (3 h)
12:00-18:00		Confinement (2 h)				
18:00-06:00	Stroboscope (1 h)	Illumination (12 h)	White noise (15 h)	Stroboscope (15 h)	Illumination (12 h)	

placed on a wooden panel and a vibration device was contacting the panel). Each period of exposure to the stressors lasted 2 to 16 h each week (Table 1). Fecal pellet output (FPO) was measured 3 times per week during the water bedding stress session of the CVS protocol to investigate stress-induced colonic motility changes. For the water bedding stress, rats in the CVS group were placed in the empty cage with only room temperature water for 1 h in the morning of Monday, Wednesday, and Friday each week. The height of the water was approximately 1-2 cm, enough to cover their feet. Fecal pellets found in the cage were counted at the end of each water bedding stress session.

Measurement of adrenal weight and radioimmunoassay of adrenocorticotropic hormone and corticosterone

All rats were killed by decapitation and blood samples were obtained. The wet weight of adrenal glands was measured. Plasma adrenocorticotropic hormone (ACTH) and corticosterone levels were measured with a commercially available kit (ELSA-ACTH, CIS Bio international, Gif-sur-Yvette Cedex, France; Coat-a-Count rat corticosterone, Diagnostic Product Cooperation, LA, CA, United States) by radioimmunoassay (RIA). The sensitivity of the assay was 2.0 pg/mL for ACTH and 5.7 ng/mL for corticosterone. The intra-/interassay coefficient of variation was 5.3%/6.1% for ACTH and 12.2%/14.9% for corticosterone.

Western blot analysis

The expression levels of transforming growth factor-β (TGF-β), IL-2, and IFN-γ were measured in the distal part of colonic tissues and plasma using western blot analysis. The distal parts of the colonic tissues were washed with Tris buffered saline (TBS), and approximately 5 g of tissue was added to lysis buffer [25 mmol/L Tris-Cl, 1 mmol/L ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 1 mmol/L dithiothreitol, 0.1% Triton X-100, protease inhibitor cocktail, and phosphatase inhibitor cocktail; pH 7.4] and homogenized. The samples were then centrifuged at 12000 *g* for 30 min, and the cytosolic fractions were obtained. Whole blood samples were centrifuged at 3000 r/min for 15 min, and the plasma was obtained and diluted with TBS. The protein concentration of both samples from the plasma and colonic tissues was estimated using bovine serum albumin as a standard and adjusted to examine identical amounts of total protein. The sample buffer was added to the samples, the mixture was boiled at 100 ℃ for 5 min, and electrophoresis was performed at 100 V for 2 h using a minigel electrophoresis apparatus (mini-PROTEIN Tetra cell; Bio-Rad Laboratories, Inc., Hercules, CA, United States). After electrophoresis, the gels were stained with Coomassie brilliant blue R-250 for 1 h. Following staining, the samples were destained with 10% acetic acid and 10% methanol, and the protein bands were observed. The proteins were transferred to 0.45 μm polyvinylidenedifluoride membranes (Roche Diagnostics GmbH, Mannheim, Germany) using a protein transfer apparatus (mini Transblot cell; Bio-Rad Laboratories) at 100 V for 90 min. To prevent non-specific binding of the primary antibody, the polyvinylidenedifluoride membranes were incubated with blocking buffer (5% skim milk in Tris buffer saline with 0.05% tween 20 (TBST, pH 7.6) for 1 h. The membranes were washed 3 times with TBST and incubated overnight at 4 ℃ with primary antibodies (TGF-β, IL-2, and IFN-γ) diluted to 1:1000 with TBS containing 3% bovine serum albumin. The membranes were then washed 4 times with TBST. Next, the membranes were incubated for 1 h with a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Enzo Life Science International Inc., Plymouth Meeting, PA) (1:5000). The membranes were washed 4 times with TBST, incubated with Immobilon Western chemiluminescent horseradish peroxidase substrate (Millipore., MA, United States), and exposed to a chemiluminescence film in a dark room. The expression levels of TGF-β, IL-2, and IFN-γ were compared. In addition, as a control experiment, double staining for glyceraldehyde-3-phosphate dehydrogenase was performed with TGF-β, IL-2, and IFN-γ under identical conditions.

Statistical analysis

All values are represented as the mean ± SE. Statistical analyses were performed out with SPSS software (V.18.0 SPSS Inc., Chicago, IL, United States). One-way analysis of variance (ANOVA) was performed, followed by Bonferroni post-hoc tests to analyze changes in all parameters between all groups. For comparing fecal pellet output between the CVS-A and CVS-B groups, the data were analyzed using repeated measures ANOVA and unpaired Student's *t* tests. Null hypotheses of no differences were rejected if *P* values were less than 0.05.

RESULTS

Sweet food preference test

All rats initially preferred sweet food to chow food at first and some rats even consumed 100% sweet food.

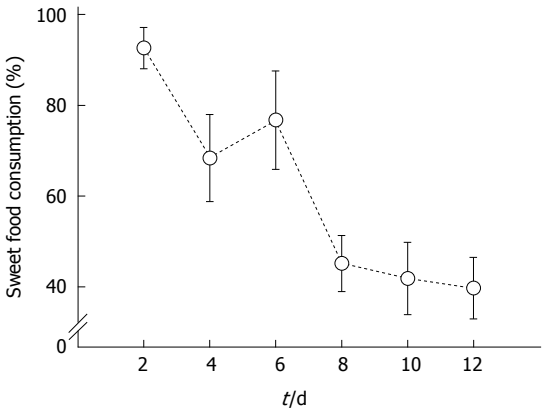

Figure 1 Time-dependent changes of sweet food consumption in normal rats for 12 d. The percentage of sweet food consumption decreased to below 50% 8 d after sweet food feeding.

However, the consumption rate of sweet food was gradually decreased and reduced to below 50% of total intake eight days after sweet food feeding (Figure 1).

Changes in body weight and food intake

At the beginning of the stress experiment, no significant differences were observed in body weight between all groups (CON, 292.50 ± 5.16 g; CVS-A, 291.08 ± 4.73 g; CVS-B, 292.07 ± 2.06 g). However, one week after beginning the stress protocol, there was a significant difference in body weight and food intake between the CON and other groups (Figure 2A, B).

The body weight (BW) of the stress groups (CVS-A, 361.08 ± 4.77 g; CVS-B, 354.13 ± 7.76 g) were significantly lower than that of the control group (399.22 ± 11.12 g) throughout the remainder of the experimental period (Figure 2A, $P < 0.05$). However, there was no difference in BW between the CVS-A and CVS-B groups (Figure 2A). From the second week onward, the amount of consumed food and total calorie intake became significantly lower in the CVS-B group compared with the other groups (Figure 2B, C).

In contrast to the result of the preferential test in normal rats, the total consumption rate of sweet food was maintained between 60% and 80% in the CVS-B group until the end of the experimental period (Figure 2D).

Accumulated FPO

The CVS-B group barely defecated except during the first week, whereas the CVS-A group continuously expelled fecal pellets during water bedding throughout the entire experimental period. As a result, there was a significant difference in the accumulated FPO between the CVS-A and CVS-B groups from the second week onward (Figure 3). Data were analyzed with repeated-measures ANOVA. Mauchly's test of sphericity was significant, and therefore, the Greenhouse-Geisser correction was used. The repeated measures ANOVA revealed highly significant differences in FPO between groups [F (1, 10) = 6.989, $P = 0.025$] and time [F (1, 10) = 10.657, $P = 0.003$)] on water bedding stress. The results also demonstrated a significant interaction between group and time [F (1, 10) = 5.154, $P = 0.028$], thus indicating that the CVS-A rats increased FPO differently than the CVS-B rats (Figure 3).

Adrenal gland weight and plasma corticosterone and ACTH levels

The adrenal weight-to-BW ratio was significantly higher in the stress groups (CVS-A, 0.14 ± 0.01; CVS-B, 0.14 ± 0.01) than in the control group (0.12 ± 0.01) (Figure 4A, $P < 0.05$).

The plasma corticosterone and ACTH levels were significantly higher in the CVS-A (537.42 ± 32.95, 44.44 ± 6.54 pg/mL) and CVS-B (655.07 ± 30.82, 65.46 ± 4.44 pg/mL) groups than in the control group (46.96 ± 13.29, 8.51 ± 1.35 pg/mL) (Figure 4B, C, $P < 0.05$). Notably, the ratio of corticosterone to ACTH was significantly increased in the CVS-A group only (Figure 4D, $P < 0.05$).

Western blot analysis of anti- and pro-inflammatory cytokines in the plasma and the distal colon

There was no difference in TGF-β expression in the plasma and colon between all groups. Rats exposed to CVS displayed significantly increased expression of IL-2 and IFN-γ in the plasma and distal colon compared to the control group, whereas this effect was significantly attenuated in the CVS-B group (Figure 5, $P < 0.05$).

DISCUSSION

In humans, chronic stress can induce increased comfort food intake[14]. In addition, chronically stressed animals increase their ingestion of palatable food when given a choice of highly palatable food, such as lard or sugar[15,17,19] possibly decreasing the stress response in the HPA axis[17].

Therefore, we hypothesized that eating sweet food might affect the HPA axis and reduce chronic stress effects through CRF stabilization in the hypothalamus, with beneficial effects on abnormal changes of colonic motility and colonic inflammatory cytokine profiles in rats. The main findings of this study on concurrent sweet food ingestion under chronic stress are that (1) the stress-induced FPO increment reflecting increased colonic motility was reduced; (2) the corticosterone/ACTH ratio was reduced to control levels; and (3) the pro-inflammatory cytokines increased by CVS were attenuated.

Preference for sweet food

The consumption rate of sweet food in the normal rats gradually decreased and reduced below 50% of total intake eight days after sweet food feeding. In contrast to normal rats, the consumption rate of sweet food ranged from 60% to 80% of total intake in stressed rats

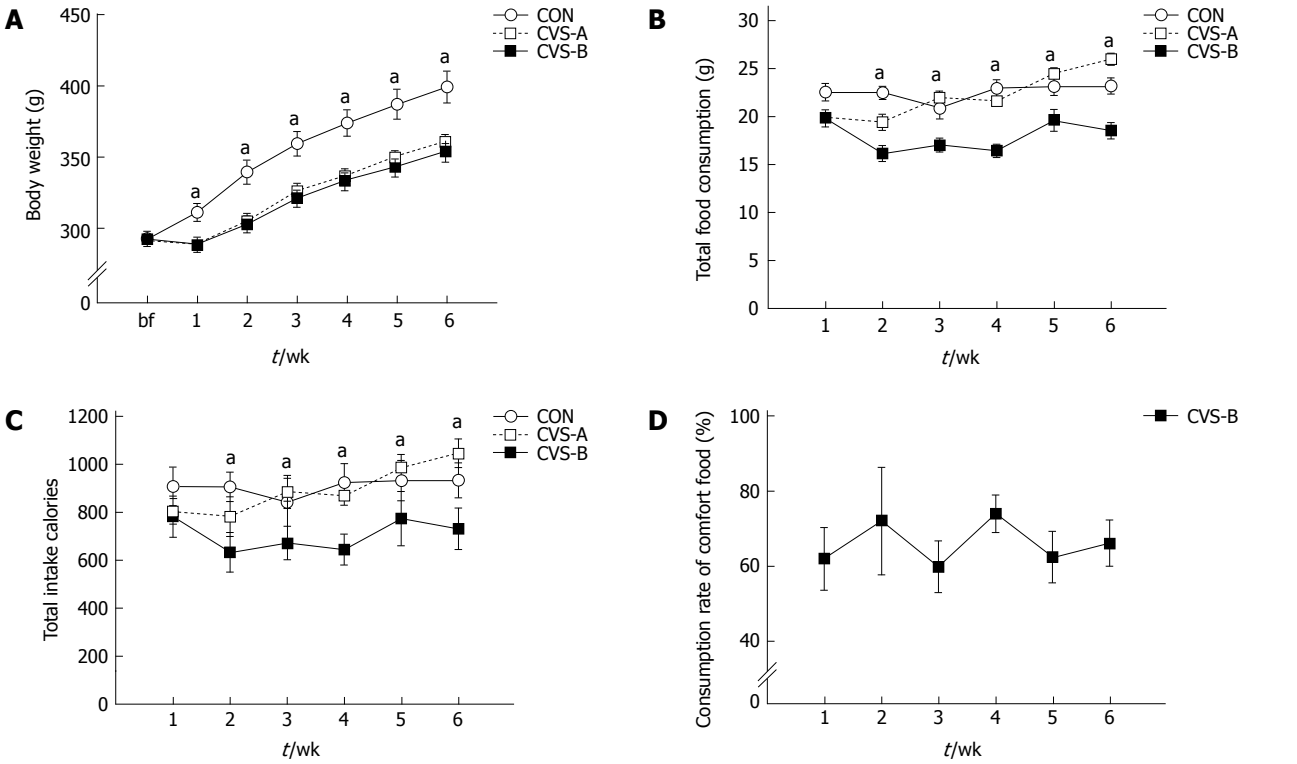

Figure 2 Time-dependent changes of body weight (A), food consumption (B), caloric efficiency (C), and consumption rate of sweet food (D) in each group for 6 wk. There were significant differences in body weight between control and treated groups. Total food consumption and caloric intake were significantly reduced in the chronic variable stress-B (CVS-B) group only. Denotes significant differences among groups based on one-way analysis of variance and the Bonferroni test ($^aP < 0.05$ *vs* other groups).

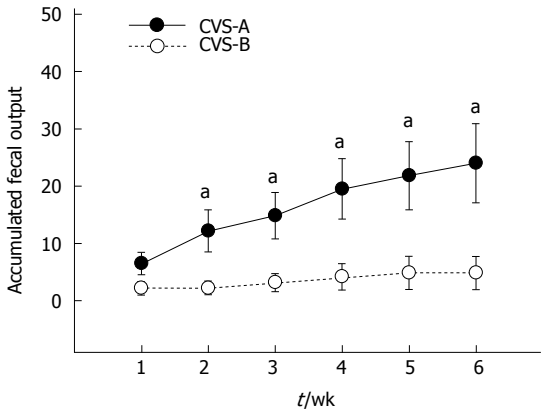

Figure 3 Accumulated fecal pellet output in the chronic variable stress-A and chronic variable stress-B groups. There was no significant difference in the first week, but accumulated fecal output difference gradually increased with time. Statistical analysis was performed using repeated measures ANOVA and unpaired *t* test in each week between groups ($^aP < 0.05$ *vs* chronic variable stress (CVS)-B group).

through the entire experimental period without decrement. The decrement of sucrose intake observed in normal rats in this study may be related to the concomitant increment of insulin and leptin levels with increasing adiposity[20].

A plausible explanation for the increased ingestion of sweet food in the CVS rats may be related to the reduction of anxiety through the activation of the reward system. It has also been suggested that this preference is likely because of a higher level of anxiety in stressed rats because the preference for sweet food in rats under restraint stress could be reversed by diazepam[17]. In addition, eating palatable food, as with drug abuse, can activate the brain reward system, comprised of opioid, dopamine, and endocannabinoid[21].

Accumulated FPO

The monitoring of FPO is used to measure stress response in animal experiments[22,23]. We quantified FPO during water bedding stress in the present study to demonstrate the effect of sweet food on stress-induced colonic motility. Cumulative FPO was significantly higher in the CVS-A group compared with the CVS-B group throughout the entire experimental period except for the first week.

Psychological stress activates some areas in the CNS including the paraventricular nucleus (PVN) and induces rapid transcription of the gene encoding CRF in the PVN, resulting in the stimulation of the sacral parasympathetic nucleus, which innervates the descending colon[1]. Foster *et al*[24] recently demonstrated that chronic access to sucrose or lard prior to the stress period significantly decreased CRF mRNA expression in the PVN after restraint stress in rats. Based on their report, we can speculate that sweet food might decrease CRF production in the PVN during stress periods and consequently prevent stress-induced colonic hyper-motility. The evidence across these studies leads to the interpretation that the FPO difference between the CVS-A and CVS-B groups in this study may result from a decreased level of

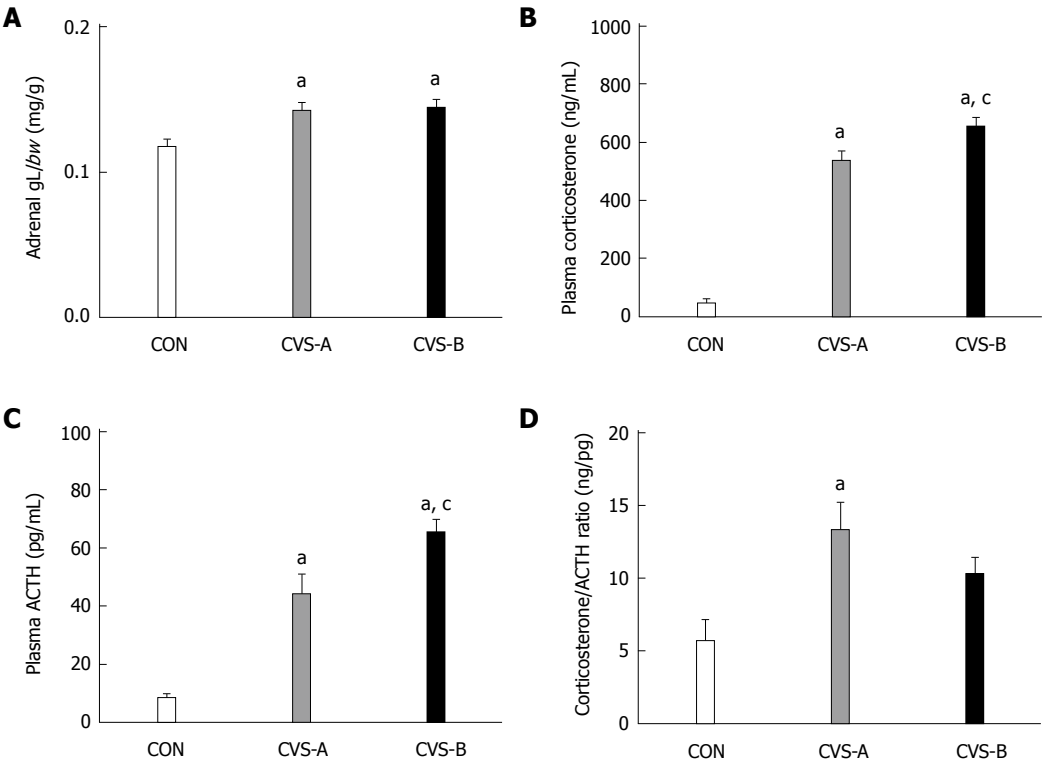

Figure 4 Relative weight of the adrenal gland (A), plasma corticosterone levels (B), ACTH levels (C), and the ratio of corticosterone/ACTH following the 6-wk stress experiment. Denotes significant difference between control and other groups ($^{a}P < 0.05$ *vs* control groups). There was a significant difference in plasma corticosterone and adrenocorticotropic hormone (ACTH) concentrations between CON and the other groups. Denotes significant difference between chronic variable stress (CVS)-A and CVS-B group ($^{c}P < 0.05$ *vs* CVS-A groups). There was a significant difference in plasma corticosterone and ACTH concentration between CVS-A and CVS-B groups. CVS: Chronic variable stress.

CRF in the PVN through the effect of sweet food.

Alteration of the HPA axis

Previous reports regarding alterations of the HPA axis in IBS patients are not consistent because of differing methodologies, patient populations, sexes, and comorbid psychiatric conditions[6]. Additionally, the alteration of ACTH and corticosterone depends on the mode and duration of stress[25]. In our study, both of the CVS groups displayed increased ACTH and corticosterone levels. Because FPO, the well-known stress response, was decreased by sweet food intake in the CVS-B group, we expected a relatively normal function of the HPA axis in the CVS-B group compared with the CVS-A group. Contrary to our expectations, the levels of ACTH and corticosterone were higher in the CVS-B group compared with the CVS-A group. However, the ratio of corticosterone/ACTH increased in the CVS-A group, but was not significantly different between the CVS-B and control groups. Chang *et al*[6] recently reported that basal levels of plasma ACTH significantly decreased while the level of cortisol tended to increase in IBS patients using 24 h blood sampling. This finding might be interpreted as enhanced adrenocortical sensitivity to ACTH and is similar to the corticosterone/ACTH ratio of the CVS-A group in our experiment. In depressive patients, plasma ACTH responses to stress and CRF administration are blunted, whereas plasma cortisol responses are normal or augmented[26,27], suggesting down-regulation or desensitization of CRF receptors in the pituitary glands and adrenal hyper-responsiveness to ACTH[28,29]. Thus, the higher corticosterone/ACTH ratio in the CVS-A group could be explained by enhanced adrenocortical sensitivity to ACTH or down-regulation of the CRF receptor at the hypothalamus by the high CRF milieu in the CNS, resulting in relatively low ACTH levels[30,31]. The last assumption is supported by the stabilization of FPO during water bedding stress in the CVS-B group in our study and the important role of CRF in the increased colonic motility induced by stress.

Inflammatory cytokines

Corticosterone (or glucocorticoids), the end products of HPA axis activity, inhibits lymphocyte proliferation and cytotoxicity and the secretion of TNFα, IL-2, and IFN-γ[13,14]. Corticosterone also enhances the synthesis of TGF-β, another cytokine with potent anti-inflammatory activities in human T cells[32] and increases the secretion of IL-10 and IL-4 that aid in the anti-inflammatory reaction[33-35].

In this study, to investigate the difference of cytokine expression in each group, Western blot of plasma IL-2, IFN-γ and TGF-β were performed in the plasma and distal segment of the colon. Although there was no difference in TGF-β among all groups, there was an observable increase of IL-2 and INF-γ levels in both the plasma and distal colon in the CVS-A group only. The CVS-A group, exposed to CVS regarded as unpredict-

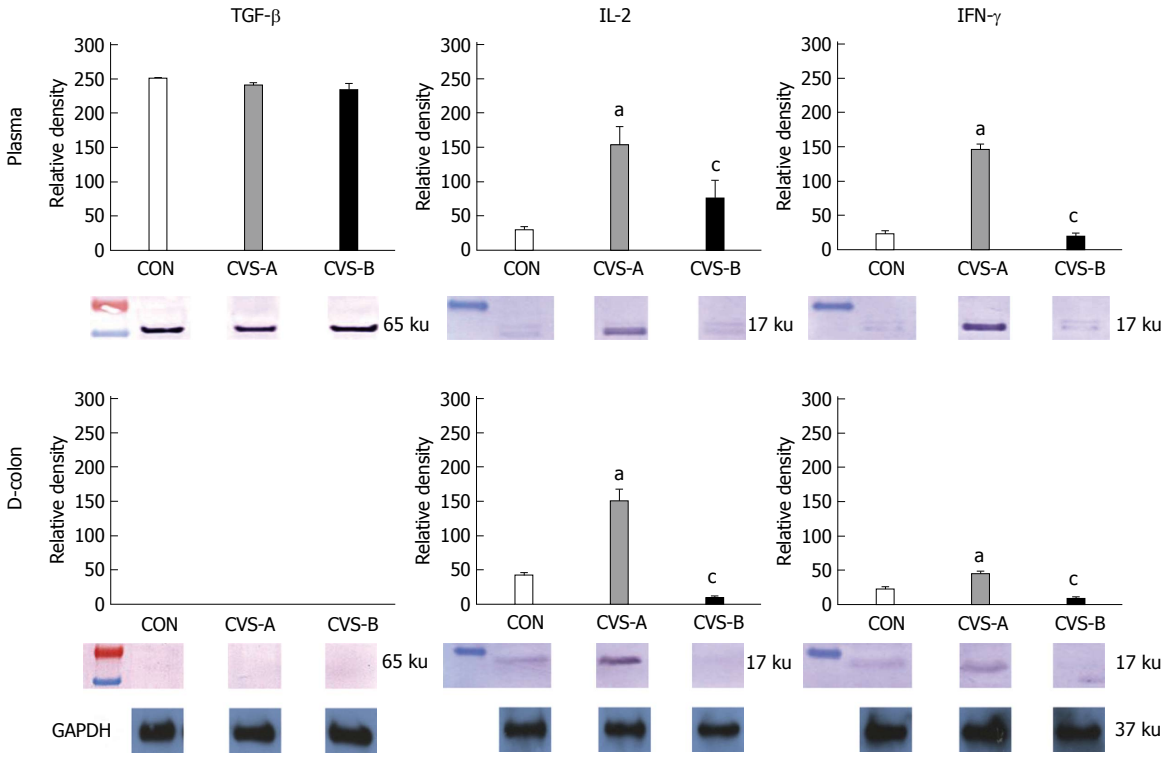

Figure 5 Inflammatory cytokine expressions in the plasma and in the distal colon by western blot analysis following the 6 wk stress experiment. Note the prominent expressions of interleukin-2 (IL-2) and interferon-γ (IFN-γ) in the distal colon and in the plasma in the chronic variable stress (CVS)-A group only. [a]Denotes significant difference between control and other groups ([a]$P < 0.05$). There was a significant difference in the relative density of IL-2 and IFN-γ in the plasma and distal colon between CON and the other groups. [b]Denotes significant difference between CVS-A and CVS-B group ([c]$P < 0.05$ *vs* CVS-A groups). There was also a significant difference in the relative density of IL-2 and IFN-γ in the plasma and distal colon between CVS-A and CVS-B groups.

able and non-adaptable stress with chow food, displayed an increase in pro-inflammatory cytokines that mediate inflammation, implying that corticosterone did not work properly in the CVS-A group.

Corticosterone is responsible for many quantitative and qualitative changes in immune function. The greatest effect of stress on the immune system is related to the suppression of immune functions and the exacerbation of diseases such as asthma, allergic, autoimmune and inflammatory diseases[36-38]. IBS is characterized as increasing HPA activity and the production of pro-inflammatory cytokines in human[5] and animal models[39]. Notably, previous studies have demonstrated that INF-γ increases in the rat colon and blood due to repeated water avoidance stress[39] or maternal separation[40], commonly used stress protocols for IBS animal studies[41]. The CVS-A group in our study also displayed increased INF-γ levels in both the plasma and colonic tissue. It has been suggested that the CRF-induced secretion of cortisol in IBS patients could be linked to the coincident increase in cytokine release and an associated decrease in the sensitivity of glucocorticoid receptors[5]. Thus, stress-induced illnesses such as IBS could be characterized by an over-activation of the HPA axis, an increase of pro-inflammatory cytokines, a change in adrenal responsiveness to ACTH[42] and possibly decreased sensitivity at the level of the glucocorticoid receptor[36,43]. In contrast to the CVS-A group, there was little expression of pro-inflammatory cytokines in the CVS-B group, which was given sweet food. The CVS-B group also exhibited a relatively normal corticosterone/ACTH ratio and colonic motility suggesting stabilization of CRF production in the hypothalamus or modulation of the HPA axis by palatable food ingestion[44]. Therefore, it can be assumed that palatable sweet food could normalize HPA dysfunction during chronic unpredictable stress periods and possibly maintain glucocorticoid receptor sensitivity. As a result, cytokine imbalance did not occur in the CVS-B group.

In conclusion, our results indicate that CVS may increase colonic motility, blunt ACTH response with over-activation of the HPA axis, decrease glucocorticoid sensitivity and increase pro-inflammatory cytokine production. These responses could be reduced by palatable sweet food consumption possibly through CRF stabilization at the central level. These results imply that reducing the effect of stress appropriately by any means is important for preventing GI symptoms in a patient with IBS.

COMMENTS

Background

Eating behavior is closely related to stress and some people preferentially consume palatable food such as chocolate or ice cream during stressful situations. Several reports have supported the hypothesis that palatable foods have been used as an ameliorator for psychological stress through the stabilization of corticotropin-releasing factor (CRF) in the hypothalamus. It seems that the

consumption of sweet food has beneficial effects on the stress-induced physiologic dysfunction. However, it has never been reported whether eating sweet food has an effect on the disordered gastrointestinal (GI) motility and immune status in chronically stressed rats.

Research frontiers

They hypothesized that sweet food may have a beneficial effect on stress induced irritable bowel syndrome (IBS)-like symptoms such as hyper-motility in rat model under chronic stress. This is the first report showing that palatable sweet food supplement can reduce colonic hyper-motility and cytokine imbalance during stress period.

Innovations and breakthroughs

This results showed that chronic stress increased colonic motility, blunted adrenocorticotropic hormone (ACTH) response with over-activation of hypothalamic-pituitary-adrenal axis, decreased the glucocorticoid sensitivity and increased pro-inflammatory cytokine production. These responses could be reduced by palatable sweet food eating possibly through CRF stabilization at the central level.

Applications

The present study showed that concurrent palatable sweet food ingestion during chronic stress reduced the stress-induced colonic hyper-motility and pro-inflammatory cytokine productions in rats. These findings provided a therapeutic implication of reducing stress for preventing GI symptoms of functional gastrointestinal disorder.

Terminology

The chronic variable stress (CVS) model (also known as the chronic mild stress model) was originally used in psychiatric research for studying depression. Rats in the CVS model are subjected to a variety of stressors, and the essential feature of this model is the variety and unpredictability of stressors. Because patients with IBS experience levels of depression between groups of psychiatric and healthy controls, CVS protocol may be a good tool for investigating the relationship between chronic stress, depression and IBS.

Peer review

Nice study showing the positive influence of sweet food on stress measured by fecal output and corticosterone/ACTH ratio.

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BRIEF ARTICLE

Procalcitonin, and cytokines document a dynamic inflammatory state in non-infected cirrhotic patients with ascites

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Abstract

AIM: To quantitate the simultaneous serum and ascitic fluid levels of procalcitonin and inflammatory markers in cirrhotics with and without ascites.

METHODS: A total of 88 consecutive severe cirrhotic patients seen in a large city hospital liver clinic were studied and divided into two groups, those with and without ascites. Group 1 consisted of 41 cirrhotic patients with massive ascites, as demonstrated by necessity for therapeutic large-volume paracentesis. Group 2 consisted of 47 cirrhotic patients without any clinically documented ascites to include either a recent abdominal computed tomography scan or ultrasound study. Serum and ascitic fluid levels of an array of inflammatory markers, including procalcitonin, were measured and compared to each other and a normal plasma panel (NPP).

RESULTS: The values for inflammatory markers assayed in the serum of Groups 1 and 2, and ascitic fluid of the Group 1. The plasma levels of the inflammatory cytokines interleukin (IL)-2, IL-4, IL-6, IL-8, interferon gamma (IFNγ) and epidermal growth factor (EGF) were all significantly greater in the serum of Group 1 as compared to that of the serum obtained from the Group 2 subjects (all $P < 0.05$). There were significantly greater serum levels of IL-6, IL-8, IL-10, monocyte chemoattractant protein-1, tumor necrosis factor-α, vascular endothelial growth factor and EGF when comparing Group 2 to the NPP. There was no significant difference for IL-1A, IL-1B, IL-2, IL-4 and IFNγ levels between these two groups. Serum procalcitonin levels were increased in cirrhotics with ascites compared to cirrhotics without ascites, but serum levels were similar to ascites levels within the ascites group. Furthermore, many of these cytokines, but not procalcitonin, demonstrate an ascites-to-serum gradient. Serum procalcitonin does not demonstrate any significant difference segregated by liver etiology in the ascites group; but ascitic fluid procalcitonin is elevated significantly in car-

diac cirrhosis/miscellaneous subgroup compared to the hepatitis C virus and alcoholic cirrhosis subgroups.

CONCLUSION: Procalcitonin in the ascitic fluid, but not in the serum, differentiates between cirrhotic subgroup reflecting the dynamic interplay of ascites, bacterial translocation and the peri-peritoneal cytokine.

Key words: Ascites; Bacterial translocation; Inflammatory markers; Procalcitonin; Cirrhosis

Core tip: Procalcitonin received much attention as a serum marker in differentiating sepsis from systemic inflammatory response syndrome and bacterial sepsis. Procalcitonin significance in assessing ascitic fluid inflammation and/or infection is less well characterized. This study demonstrates that non-infected cirrhotics with ascites *vs* those without ascites manifest peri-peritoneal based immune response mediated by a constellation of pro- and anti-inflammatory protein markers and procalcitonin. This peri-peritoneal response is distinct from the systemic immune response to the underlying hepatic disease process. Its recognition can potentially determine the likelihood for future adverse events like spontaneous bacterial peritonitis, the hepato-renal syndrome and impending death.

Attar BM, Moore CM, George M, Ion-Nedelcu N, Turbay R, Zachariah A, Ramadori G, Fareed J, Van Thiel DH. Procalcitonin, and cytokines document a dynamic inflammatory state in non-infected cirrhotic patients with ascites. *World J Gastroenterol* 2014; 20(9): 2374-2382 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i9/2374.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i9.2374

INTRODUCTION

Cirrhosis is characterized by an extensive fibronodular replacement of the hepatic parenchyma resulting in both synthetic dysfunction and portal hypertension. This process is driven by hepatic inflammatory processes in the setting of an inciting agent, such as hepatitis C virus (HCV), alcohol (ETOH) abuse or a metabolic derangement in a host with a particular genetic constitution[1-4]. Decompensated cirrhosis is identified by the presence of portal hypertensive clinical sequelae, namely hepatic encephalopathy, varices, and most commonly, ascites. Ascites is associated with two other serious complications such spontaneous bacterial peritonitis (SBP) and hepatorenal syndrome (HRS), both of which increase morbidity and can lead to death[4,5].

Bacterial translocation (BT) is the migration of enteric flora into mesenteric lymph nodes or other extra-intestinal sites[6-8]. It is well known that cirrhotics, with and without ascites, have many factors which predispose to enhanced BT including: malnutrition, altered enteric flora species and bacterial overgrowth, increased bowel stasis, altered gut permeability, and decreased mucosal defense mechanisms[8-12]. In particular, portal hypertension can lead to enteric mucosal edema and microvascular stasis, both of which further alter gut permeability[11,12]. Consistent with these facts, it known that oral medications such as cisapride and trimethoprim-sulfamethoxazole can attenuate bacterial overgrowth, enhance mucosal function, and increase intestinal transit, all leading to reduced rates of BT[13-15].

Even in the absence of overt infection, there is a significant inflammatory response in cirrhotic patients occurring as a result of a complex interaction of pro- and anti-inflammatory cytokines consisting of numerous interleukins, tumor necrosis factor-alpha (TNF-α), interferon gamma (IFNγ) and complement proteins[16-18]. This concerted cytokine response has both temporal and site specific properties in cirrhotic patients with portal hypertension[16,17]. One particular inflammatory marker is procalcitonin (PCT), a 116 amino acid protein made in multiple sites of the body including liver and intestine. PCT has received much attention as a possible discriminatory serum marker in differentiating sepsis from systemic inflammatory response syndrome (SIRS) and bacterial sepsis from non-bacterial sepsis in multiple populations including cirrhotic patients[19-27]. Its significance in measuring ascitic fluid inflammation and/or infection, *e.g.*, SBP, is less well characterized[28].

It has been shown that many of these same cytokines are generated by activated macrophages, neutrophils, NK cells and lymphocytes in peri-peritoneal tissues in cirrhotic patients[29-34]. These immune cells are activated upon sensing appropriate bacterial antigens, such as bacterial DNA or lipopolysaccharide, either in the intestinal mucosa or after BT. These interactions can also activate the adaptive immune system beyond innate infection cellular control mechanisms[33-35]. As the enteric mucosa is continuously exposed to bacterial antigens, which is enhanced strain in the setting of cirrhosis and portal hypertension, this immune response is continuously engaged and tightly regulated.

Thus, the aims of the present investigation were to: (1) quantitate the simultaneous serum and ascitic fluid levels of PCT and an array of inflammatory markers in non-infected cirrhotic patients with and without ascites; and (2) further segregate serum and ascitic fluid PCT with respect to different etiologies of liver disease.

MATERIALS AND METHODS

Subjects

A total of 88 consecutive severe cirrhotic patients seen in a large city hospital liver clinic were studied and divided into two groups. The inclusion criteria consisted of age > 18 years or > 65 years, cirrhosis documented by clinical examination, computed tomography (CT), or liver biopsy. The only exclusion criteria was the presence of any clinical infection. Group 1 consisted of 41

Table 1 Characteristics of the 88 patients studied

Primary liver disease	ETOH	NASH	HCV	Cardiac/miscellaneous	Total
Patients					
Ascites (+)	21	0	8	12	41
Ascites (–)	13	11	23	0	47
Age (yr)					54 ± 11
Ascites (+)	50 ± 1	58 ± 5	54 ± 10	55 ± 11	
Ascites (–)	48 ± 5	56 ± 8	53 ± 9	56 ± 12	
Gender (M/F)					66/22
Ascites (+)	11/9	N/A	14/9	9/8	
Ascites (–)	8/3	3/8	4/4	N/A	
MELD score					9.6 ± 3.5
Ascites (+)	9.6 ± 2.1	9.3 ± 1.6	10.3 ± 5.0	8.6 ± 2.5	
Ascites (–)	9.4 ± 2.2	9.2 ± 2.0	10.0 ± 4.6	8.6 ± 3.1	
CTP score					5.6 ± 1.2
Ascites (+)	5.8 ± 1.5	5.3 ± 0.6	5.6 ± 1.5	5.4 ± 0.8	
Ascites (–)	5.6 ± 2.0	5.3 ± 0.8	5.6 ± 1.2	5.3 ± 1.2	

Data given as mean ± SE. ETOH: Alcohol; NASH: Non-alcoholic steatohepatitis; HCV: Hepatitis C virus; MELD: Model for End-stage Liver Disease; CTP: Childs-Turcotte-Pugh.

cirrhotic patients with massive ascites, as demonstrated by necessity for therapeutic large-volume paracentesis (LVP) because of tense abdominal distension resulting in difficulty ambulating and performing the activities of normal daily living. In each case a minimum of 2 liters of ascites was removed and the maximum volume removed from any patient was 6 liters. No patient received antibiotics before the paracentesis. Group 2 consisted of 47 cirrhotic patients without any clinically documented ascites to include either a recent abdominal CT scan or ultrasound study. No patient demonstrated symptoms or clinical evidence of active infection, and no ascitic fluid from the LVP performed on Group 1 patients met criteria for SBP. SBP is diagnosed when the ascitic fluid absolute polymorphonuclear cell count is 250 cells/mL or more in the absence of recent surgery. SBP occurs in 30% of patients with ascites and has a 20% mortality rate[36].

Investigative procedures

Procalcitonin (PCT) was assessed utilizing the Bio Merieux Inc. Vidas Assay (Lombard, IL, United States) in the serum of both groups and in the ascitic fluid of Group 1. A panel of cytokines consisting of interleukin (IL)-1A, IL-1B, IL-2, IL-4, IL-6, IL-10, monocyte chemotactic protein (MCP)-1, TNF-α, IFNγ, vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF) were assayed in the serum of patients in Groups 1 and 2 and in the ascitic fluid of those in Group 1 utilizing biochip platforms obtained from Randox Life Sciences (Boston, MA, United States). Values for each assay were compared to the results obtained using a commercially available normal plasma panel (NPP) obtained from Bioreclamation, LLC (Liverpool, NY, United States).

Regulation

All 88 subjects studied agreed to be studied after reading and signing an informed written consent which was approved by the Institutional Review Board (IRB) of the Cook County Health and Hospitals system, Chicago, Illinois, United States. Additional funding was provided by a grant from the Rush-Cook County joint research endeavor program.

Statistical analysis

All data are reported as mean values ± standard error of the mean + SE). Unpaired *t* tests, χ^2 and ANOVA were used to compare the values between groups and against the NPP utilizing a P value < 0.05 as the measure of statistical significance.

RESULTS

The 41 patients in Group 1, *i.e.*, cirrhotics patients with large-volume ascites, included 21 with ETOH cirrhosis, 12 with cardiac cirrhosis/miscellaneous, 8 with extrahepatic malignancy, and 8 with HCV cirrhosis. Of this latter group, 3 also had a hepatoma (Table 1). The 47 patients of Group 2, *i.e.*, cirrhotic patients without ascites, consisted of 23 with HCV cirrhosis, 13 with ETOH cirrhosis, and 11 with non-alcoholic steatohepatitis (NASH) cirrhosis (Table 1). Further data as regards to age, gender, Model for End-stage Liver Disease (MELD) score, and Childs-Turcotte-Pugh (CTP) score are also listed in Table 1. In regards to cirrhotic etiology, the only differences were the finding of NASH patients in Group 2 compared to none in Group 1, and patients with malignancy and cirrhosis in Group 1 not present in Group 2. There were no significant differences in age, MELD score or CTP score between the two groups.

The biochemical characteristics of both groups are shown in Table 2. Pertinently, Group 1 demonstrated significantly greater prothrombin times and the international normalized ratio (INR). Group 2 demonstrated significantly higher levels of serum alanine transaminase and albumin. The characteristics of the ascitic fluid in the Group 1 are shown in Table 2. All 41 of these patients were culture negative and had significantly lower

Table 2 Biochemical data of the 88 patients

Parameter	Ascitic Group 1 (n = 41)	Non-ascitic Group 2 (n = 47)	*P* value
Hemoglobin (g/dL)	10.8 ± 0.4	10.4 ± 1.7	NS
WBC (cells × 10^3/μL)	8.3 ± 1.3	6.7 ± 2.8	NS
Platelets (cells × 10^3/μL)	132 ± 13	157 ± 66	NS
Prothrombin time (s)	20.5 ± 1.2	17.7 ± 1.1	< 0.05
INR	1.8 ± 0.1	1.5 ± 0.1	< 0.05
BUN (mg/dL)	13.3 ± 7.2	21 ± 4	NS
Creatinine (mg/dL)	1.0 ± 0.7	0.9 ± 0.2	NS
Total bilirubin (mg/dL)	5.9 ± 1.0	4.7 ± 1.9	NS
AST (IU/L)	110 ± 40	98 ± 15	NS
ALT (IU/L)	66 ± 8	210 ± 111	< 0.05
Albumin (g/dL)	2.2 ± 0.1	2.6 ± 0.1	< 0.05
Ascitic fluid WBC (cells × 10^3/μL)	417.7 ± 172.5	N/A	N/A
Ascitic fluid PMN (cells × 10^3/μL)	196.9 ± 144.2	N/A	N/A
Ascitic fluid albumin (g/dL)	0.90 ± 0.14	N/A	N/A
Ascitic fluid culture	All negative	N/A	N/A

Data given as mean ± SE. INR: International normalized ratio; BUN: Blood urea nitrogen; AST: Aspartate transaminase; ALT: Alanine transaminase; PMN: Polymorphonuclear cells; NS: Non-significant; WBC: White blood cells.

Table 3 Serum and ascitic fluid levels of inflammatory markers in the 88 patients studied

	Ascites Group 1			Non-ascitic Group 2	Control		*P* value	
	Serum	Ascitic fluid	*P* value	Serum	NPP	*P* value	Group 1 (serum) *vs* Group 2	Group 1 (serum) *vs* NPP
IL-1A (ng/mL)	0.21 ± 0.08	0.21 ± 0.04	NS	0.17 ± 0.07	0.09 ± 0.01	NS	NS	< 0.05
IL-1B (ng/mL)	6.15 ± 2.83	3.10 ± 0.48	NS	3.56 ± 1.11	2.38 ± 0.62	NS	NS	< 0.05
IL-2 (ng/mL)	5.61 ± 3.49	1.31 ± 0.35	< 0.05	0.96 ± 0.01	1.01 ± 0.40	NS	< 0.05	< 0.05
IL-4 (ng/mL)	2.51 ± 0.79	9.48 ± 1.21	< 0.05	1.25 ± 0.07	1.21 ± 0.39	NS	< 0.05	< 0.05
IL-6 (ng/mL)	94.43 ± 29.58	1240.5 ± 365.64	< 0.05	7.27 ± 1.93	0.64 ± 0.16	< 0.05	< 0.05	< 0.05
IL-8 (ng/mL)	111.83 ± 37.01	335.08 ± 60.0	< 0.05	50.19 ± 18.84	2.22 ± 0.79	< 0.05	< 0.05	< 0.05
IL-10 (ng/mL)	1.47 ± 0.31	57.15 ± 22.32	< 0.05	0.95 ± 0.23	0.62 ± 0.08	< 0.05	NS	< 0.05
MCP-1 (ng/mL)	141.23 ± 23.39	456.35 ± 42.03	< 0.05	200.69 ± 18.19	57.11 ± 12.59	< 0.05	< 0.05	< 0.05
TNF-α (ng/mL)	5.26 ± 1.18	26.32 ± 7.13	< 0.05	9.89 ± 3.54	1.61 ± 0.80	< 0.05	NS	< 0.05
IFNγ (ng/mL)	1.08 ± 0.38	1.61 ± 0.41	NS	0.51 ± 0.04	0.47 ± 0.34	NS	< 0.05	NS
VEGF (ng/mL)	38.31 ± 18.73	234.82 ± 103.11		55.94 ± 11.37	7.14 ± 4.06		NS	< 0.05
EGF (ng/mL)	72.42 ± 14.95	1.78 ± 0.36		45.70 ± 7.38	3.12 ± 0.99		< 0.05	< 0.05

Data given as mean ± SE. IL: Interleukin; MCP: Monocyte chemotactic protein; TNF: Tumor necrosis factor alpha; IFN: Interferon gamma; VEGF: Vascular endothelial growth factor; EGF: Epidermal growth factor; NPP: Normal plasma panel; NS: Non-significant.

albumin levels in the ascitic fluid compared to serum. The mean serum-ascites albumin gradient (SAAG) was > 1.1 g/dL in Group 1.

The serum PCT levels of Group 1 (n = 41) were significantly greater than in Group 2 (n = 47) and the NPP (0.42 ± 0.19 ng/mL *vs* 0.10 ± 0.01 ng/mL, P < 0.05); approximately × 4 greater than each group. There was not a significant difference between the serum and ascitic fluid PCT levels of Group 1 (0.42 ± 0.19 ng/mL *vs* 0.27 ± 0.13 ng/mL, P > 0.05). The ascitic fluid PCT in Group 1 was significantly greater than the serum PCT level of Group 2 and the NPP (n = 12) (0.27 ± 0.13 ng/mL *vs* 0.10 ± 0.01 ng/mL, P < 0.05); approximately × 3 greater than each group. The PCT levels between Group 2 and the NPP were not significantly different (0.10 ± 0.01 ng/mL *vs* 0.09 ± 0.01 ng/mL, P > 0.05).

Table 3 reports the values for inflammatory markers assayed in the serum of Groups 1 and 2, and ascitic fluid of the Group 1. The plasma levels of the inflammatory cytokines IL-2, IL-4, IL-6, IL-8, IFNγ and EGF were all significantly greater in the serum of Group 1 as compared to that of the serum obtained from the Group 2 subjects (all P < 0.05). However, IL-1A, IL-1B, IL-10, MCP-1, TNF-α, and VEGF serum levels were not significantly different when comparing the serum values of Groups 1 and 2 for these measures. There were significantly greater levels of IL-2, IL-4, IL-6, IL-8, IL-10, MCP-1, TNF-α, VEGF and EGF in the ascitic fluid compared to serum of Group 1. There was no significant difference for IL-1A, IL-1B, and IFNγ between these groups. There were significantly greater levels of IL-1A, IL-4, IL-6, IL-8, IL-10, MCP-1, TNF-α, VEGF and EGF in Group 1 ascites compared to NPP. IL-1B and IL-2 levels were not significantly different when comparing Group 1 ascites to the NPP (not included in Table 3). There were significantly greater levels for all inflammatory markers, except IFNγ when comparing Group 1 serum levels to the NPP. There were significantly greater serum levels of IL-6, IL-8, IL-10, MCP-1, TNF-α, VEGF and EGF when comparing Group 2 to

Table 4 Serum and ascitic procalcitonin levels segregated by liver disease

	Serum	Ascites	*P* value
EtOH (ng/mL)	0.38 ± 0.22	0.22 ± 0.13	NS
HCV (ng/mL)	0.44 ± 0.27	0.13 ± 0.12	NS
Cardiac/miscellaneous (ng/mL)	0.95 ± 0.74	0.77 ± 0.07	NS
P value	NS	< 0.05[a]	

[a]*P* < 0.05 cardiac/misc *vs* non-cardiac diseases only. Data given as mean ± SE. EtOH: Alcohol; HCV: Hepatitis C virus; NS: Not significant.

Table 5 The serum-ascitic fluid (P-A) gradient for Group 1

Serum-ascitic fluid gradient	Value
IL-1A (ng/mL)	-0.0008 ± 0.097
IL-2 (ng/mL)	4.30 ± 3.53
IL-4 (ng/mL)	-6.96 ± 1.48
IL-6 (ng/mL)	-1146.11 ± 362.15
IL-8 (ng/mL)	-223.24 ± 58.54
IL-10 (ng/mL)	-55.68 ± 22.18
MCP-1 (ng/mL)	-315.13 ± 39.24
TNF-α (ng/mL)	-21.06 ± 7.33
IFNγ (ng/mL)	-0.52 ± 0.48
VEGF (ng/mL)	196.52 ± 105.60
EGF (ng/mL)	70.64 ± 14.74
PCT (ng/mL)	-0.134 ± 0.12

Data given as mean ± SE. IL: Interleukin; MCP: Monocyte chemotactic protein; TNF: Tumor necrosis factor α; IFN: Interferon gamma; VEGF: Vascular endothelial growth factor; EGF: Epidermal growth factor; PCT: Procalcitonin.

the NPP. There was no significant difference for IL-1A, IL-1B, IL-2, IL-4 and IFNγ levels between these two groups.

The serum and ascitic fluid PCT levels segregated as to the etiology of the liver disease are shown in Table 4. The cardiac cirrhosis/miscellaneous group had the greatest PCT levels in serum being × 2-3 that present in the other three disease subgroups. The ETOH and HCV subgroups each had PCT serum levels approximately half that of the cardiac cirrhosis/miscellaneous group. These differences were not statistically significant amongst each other. All PCT serum levels in each cirrhosis subgroup were significantly greater than that in the NPP ($P < 0.05$), data not shown. The greatest ascitic fluid PCT levels were present in the cardiac cirrhosis/miscellaneous group, with the levels for the HCV and ETOH cirrhosis subgroups being 17% and 29% of that group ($P < 0.05$). The ascitic fluid PCT levels in all of the cirrhosis subgroups were greater than in the NPP ($P < 0.05$), data not shown.

Table 5 displays the mean inflammatory marker gradient (serum minus ascitic fluid) for Group 1 patients. Gradients with positive values reflect inflammatory markers in which the serum level is greater than the ascitic fluid level. Gradients with negative values reflect inflammatory markers in which the ascitic fluid level is greater than the serum level. Thus, the IL-4, IL-6, IL-8, IL-10, MCP-1, and TNF-α levels were greater in the ascitic fluid than the in serum. The IL-1A and PCT serum and ascitic fluid levels were approximately equal. The IL-2, VEGF and EGF levels were greater in the serum than in the ascitic fluid.

Utilizing linear regression analysis, no relationship between the serum or ascitic PCT levels were observed when the PCT values was analyzed against either the whole blood or ascitic fluid total white blood cell counts, number and percentage of either the monocytes or lymphocytes as well as the number and percentage of monocytes plus lymphocytes in whole blood (data not shown).

DISCUSSION

Cirrhotic patients with portal hypertension commonly develop ascites, which itself is associated with SBP, HRS and even death[1-5]. Ascitic fluid promulgation further involves the dysregulation of the renin-angiotensin-aldosterone axis and the resultant alterations in vascular tone and volume control[4,5]. These processes are known to affect enteric mucosal permeability and enhance BT. In a recursive way, BT itself and the resultant host inflammatory response can not only alter enteric mucosal permeability, but exacerbate ascites and in certain instances lead to SBP[7-9,11,12].

In infected ascites, the cellular and cytokine inflammatory response is expectantly activated. The cellular aspect is so sensitive and reliable that an ascitic fluid neutrophil count of $\geqslant 250 \times 10^3$ cells/μL on diagnostic paracentesis, even in the absence of symptoms, can provide a provisional diagnosis of SBP while awaiting ascitic fluid culture results[5]. Furthermore, in those patients not clinically responding to SBP treatment, repeat paracentesis with cell count and differential studies may be performed to provide a rapid prognostic tool[5]. Other non-cellular inflammatory markers in SBP have been studied as well, including ascitic fluid lactoferrin, with productive results[37,38].

Interestingly though, even in non-infected cirrhotic patients with portal hypertensive ascites, there seems to be a significant degree of cellular and cytokine inflammation[16-18]. Multiple studies have documented a number of pro- and anti-inflammatory markers in the serum and ascitic fluid of such patients[17,18]. Furthermore, an immune response, both innate and adaptive, can be generated and exacerbated by a network of immunocytes sampling bacterial antigens in the enteric mucosa or after BT[29-37]. These processes are in a dynamic equilibrium, wherein a particular arrangement of inflammatory markers, both in the serum and ascitic fluid, interacts with a critical load of bacterial antigen. In certain instances, for reasons not entirely clear, this immune defense mechanism can be overwhelmed and lead to enhanced BT or even SBP. Whereas in other instances, these same defense mechanisms are successful in identifying and eliminating or sufficiently suppressing the bacteria load.

Serum procalcitonin levels have been shown to be

significantly increased above the level of 0.5 ng/mL in 88% of patients with decompensated cirrhosis and proven bacterial infection. About 50% of these patients present with extremely high serum PCT levels of greater than 5 ng/mL, correlating with high rates of in-hospital mortality[21]. However, a similar increase in serum procalcitonin levels was observed in 46% of patients who presented with acute alcoholic hepatitis and underlying cirrhosis with no evidence of bacterial infection. Thirty-one percent of patients with acute viral hepatitis were also found to have an elevated serum procalcitonin. A normal level of PCT (< 0.5 ng/mL) was observed in all patients presenting with uncomplicated cirrhosis regardless of the etiology of cirrhosis. These data suggest that a serum PCT > 0.5 ng/mL is not sufficient to differentiate between liver disease patients who have a bacterial infection from those without it[21].

In contrast, Connert *et al*[22] demonstrated that serum PCT levels above 0.58 ng/mL is a valid marker of bacterial infection in decompensated cirrhotic patients with a sensitivity of 92% and specificity of 78%. Patients who present with such levels of serum PCT were associated with 50% mortality in the first two months. Interestingly, serum levels of IL-6, TNF-α, and C-reactive protein failed to discriminate the presence or absence of an associated bacterial infection[22].

A higher cut-off value of serum PCT was use by Viallon *et al*[23] to diagnose SBP in cirrhotic patients. Serum procalcitonin levels of 0.75 ng/mL and higher were diagnostic of SBP with a sensitivity of 95% and a specificity of 98%. Importantly, ascitic fluid to serum ratio of TNF-α and IL-6 was greater than 2 in all 21 patients presenting with SBP. In contrast, the ascitic fluid to serum ratio of PCT was < 1 in all SBP cases suggesting that procalcitonin is not produced intraperitoneally. Thus, serum PCT determination may play a role as a non-invasive test in the diagnosis of SBP[23].

Su *et al*[28] reviewed the current evidence on the diagnostic value of serum procalcitonin levels in identifying SBP. They included three qualifying studies consisting of 181 episodes of suspected infection with 27% being confirmed as SBP. Serum PCT levels demonstrated moderate to high accuracy for PCT as a helpful marker for SBP. However, further large and prospective studies are needed to clarify these findings[28].

In this study, an array of inflammatory markers including PCT, a family of ILs, MCP-1, TNF-α, IFNγ, VEGF and EGF were analyzed in the serum and ascites of cirrhotic patients in Group 1 and the serum of cirrhotic patients without ascites in Group 2. The PCT level was significantly greater in the serum and ascites of Group 1 patients as compared to Group 2 patients. Additionally, the serum levels of IL-2, IL-4, IL-6, IL-8, MCP-1, IFN-γ and EGF were all significantly elevated in cirrhotic patients with ascites compared to those without ascites. Furthermore, this array of inflammatory markers, except for PCT and IFNγ, but with the addition of IL-10, TNF-α and VEGF demonstrated a significant difference between the levels present in ascites and serum. Such differences define a concentration gradient in which the majority of these inflammatory markers are greater in the ascitic fluid as compared to the serum. Such a gradient suggests enhanced peri-peritoneal production and promulgation of these inflammatory markers compared to the response arising from more systemic processes.

A second aim of the study was to characterize the inflammatory response segregated by liver etiology, and for this only serum and ascitic PCT levels in cirrhotic patients with ascites were evaluated. The serum PCT level was significantly elevated compared to NPP levels, but was not statistically different compared to the ascitic fluid levels. Additionally, there was a significant difference in ascitic fluid, but not serum, PCT levels when comparing the cardiac cirrhosis/miscellaneous subgroup to other subgroups. This study also demonstrated that PCT levels have no relationship with serum or ascitic fluid total leukocyte or monocyte levels, supporting data of its inflammatory expression pattern in multiple non-hematologic organ sites[19,20]. As there was no cohort group with infection studied, no conclusion as to its discriminatory capacity between sepsis and SIRS can be made. In particular, the role of PCT as a diagnostic marker of the severity of SBP has received little investigation.

The serum-ascites gradients of these inflammatory markers lends further support to the complex peri-peritoneal interplay of BT and immunocyte activation in both non-infected and infected cirrhotic patients with ascites[16-20,29]. It is interesting to speculate on the particular importance of ascites itself. This study documents the finding in patients with requiring LVP. However, the frequency of LVP, the amount of fluid removed, and background use or failure of diuretic therapy was not documented. A number of studies have reported that the manner in which ascitic fluid is reduced, *e.g.*, either by LVP or by diuretic therapy can have significant impact upon ascitic fluid complement, immunoglobulin, and opsonic concentrations[39,40]. It is reasonable to infer that certain concentrations and combinations of these factors in the setting of BT are crucial to an appropriate inflammatory response and in controlling infection. These studies suggest that diuretic therapy maintains higher concentrations of these key proteins and might be beneficial in preventing SBP compared to LVP, with or without combined diuretics[39,40].

The patients in the present study absent any clinical infection, and specifically those with ascites had demonstrated sterile ascitic fluid documented by culture. It was not documented however as to whether these patients had suffered any recent gastro-intestinal illnesses, change in bowel habits, or change in medications or diet. Such events are known to contribute both to enteric mucosal function and integrity, bacterial burden and distribution of species[4,6,13-15]. These alterations might then have effects upon the magnitude of BT and the pattern and in-

tensity of the local inflammatory response that have not been identified in the present report.

Additionally, despite the addition of ascites to Group 1, as referenced in Table 1, these two groups of patients had very similar clinical and biochemical backgrounds, including their MELD scores, which is a logarithmic equation including serum total bilirubin, creatinine and INR[41]. In these two groups only the INR was significantly different in patients with ascites. It might be commented that in so far as ascites represents decompensated liver disease, one might expect a worse MELD score, itself a marker for 90 day mortality without liver transplant[4,5,42,43]. Yet, the MELD score, while effective, is still quite imperfect in capturing the total biology of cirrhosis. Thus, the provisional addition of "exception points" for certain patients, *e.g.*, those with hepatocellular carcinoma, who have particularly good post-transplant outcomes that would not be predicted by the traditional MELD score has been accepted[44]. Further, is the constant interest in modifying the MELD score equation itself with a serum sodium (Na) component; the so called "MELDNa" score[45]. It is likely that serum Na and albumin, in addition to volume status have a role to play in capturing quantitatively the biological essence and clinical relevance of ascites to treatment and overall clinical outcome, but this remains an area of continued research.

Based on the present study, the serum level of PCT as well as the serum level of other inflammatory cytokines assessed in this study could be used to determine the likelihood for reduced life span or future adverse events such as the development of spontaneous bacterial peritonitis or the hepatorenal syndrome in those with ascites. This however remains to be proven in longitudinal studies[28].

In summary, this study demonstrates that non-infected cirrhotic patients with ascites as compared to those without ascites manifest a unique underlying immune response as mediated by a constellation of pro- and anti-inflammatory protein markers. This immune response functions in a gradient fashion, suggesting a peri-peritoneal predominance compared to systemic origin. From this data, it is reasonably inferred that the biology of ascites has a strong role in the interplay between BT and the cellular and cytokine inflammatory response. Further studies might examine the arrangement of these inflammatory markers in response to ascitic fluid management and/or particular concentrations of enteric flora.

COMMENTS

Background

Serum procalcitonin (PCT) level has received much attention as a possible discriminatory serum marker in differentiating sepsis from systemic inflammatory response syndrome and bacterial sepsis from non-bacterial sepsis in multiple populations including cirrhotic patients.

Research frontiers

It is well known that cirrhotics, with and without ascites, have many factors which predispose to enhanced bacterial translocation (BT). The research hot spot is to quantitate the simultaneous serum and ascitic fluid levels of PCT and several inflammatory markers in non-infected cirrhotic patients with and without ascites, and whether these markers differ with respect to etiologies of liver disease.

Innovations and breakthrough

The cardiac cirrhosis/miscellaneous group had the greatest serum PCT levels. The alcohol and hepatitis C virus subgroups each had PCT serum levels approximately half that of the cardiac cirrhosis/miscellaneous group. This study demonstrates that non-infected cirrhotic patients with ascites as compared to those without ascites manifest a unique underlying immune response as mediated by a constellation of pro- and anti-inflammatory protein markers. This immune response functions in a gradient fashion, suggesting a peri-peritoneal predominance compared to systemic origin. From this data, it is reasonably inferred that the biology of ascites has a strong role in the interplay between BT and the cellular and cytokine inflammatory response. Further studies might examine the arrangement of these inflammatory markers in response to ascitic fluid management and/or particular concentrations of enteric flora.

Applications

This study results suggest that the serum level of PCT as well as the serum level of other inflammatory cytokines assessed could be used to determine the likelihood for reduced life span or future adverse events such as the development of spontaneous bacterial peritonitis or the hepatorenal syndrome in those with ascites. This however remains to be proven in longitudinal studies.

Terminology

Decompensated cirrhosis is identified by the presence of portal hypertensive clinical sequelae, namely hepatic encephalopathy, varices, and most commonly, ascites. Ascites is associated with two other serious complications such spontaneous bacterial peritonitis and hepatorenal syndrome, both of which increase morbidity and can lead to death.

Peer review

This is an article of importance in its field. This study is an innovated research with pretty good presentation and readability of the article. The authors reported an interesting study about findings of several biomarkers in cirrhotic patients with and without ascites.

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BRIEF ARTICLE

Endocrine cells in the ileum of patients with irritable bowel syndrome

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Abstract

AIM: To study the ileal endocrine cell types in irritable bowel syndrome (IBS) patients.

METHODS: Ninety-eight patients with IBS (77 females and 21 males; mean age 35 years, range 18-66 years) were included, of which 35 patients had diarrhea (IBS-D), 31 patients had a mixture of both diarrhea and constipation (IBS-M), and 32 patients had constipation (IBS-C) as the predominant symptoms. The controls were 38 subjects (26 females and 12 males; mean age 40 years, range 18-65 years) who had submitted to colonoscopy for the following reasons: gastrointestinal bleeding, where the source of bleeding was identified as hemorrhoids (n = 24) or angiodysplasia (n = 3), and health worries resulting from a relative being diagnosed with colon carcinoma (n = 11). The patients were asked to complete the: Birmingham IBS symptom questionnaire. Ileal biopsy specimens from all subjects were immunostained using the avidin-biotin-complex method for serotonin, peptide YY (PYY), pancreatic polypeptide (PP), enteroglucagon, and somatostatin cells. The cell densities were quantified by computerized image analysis, using Olympus cellSens imaging software.

RESULTS: The gender and age distributions did not differ significantly between the patients and the controls (P = 0.27 and P = 0.18, respectively). The total score of Birmingham IBS symptom questionnaire was 21 ± 0.8, and the three underlying dimensions: pain, diarrhea, and constipation were 7.2 ± 0.4, 6.6 ± 0.4, and 7.2 ± 0.4, respectively. The density of serotonin cells in the ileum was 40.6 ± 3.6 cells/mm^2 in the controls, and 11.5 ± 1.2, 10.7 ± 5.6, 10.0 ± 1.9, and 13.9 ± 1.4 cells/mm^2 in the all IBS patients (IBS-total), IBS-D, IBS-M, and IBS-C patients, respectively. The density in the controls differed significantly from those in the IBS-total, IBS-D, IBS-M, and IBS-C groups (P < 0.0001, P = 0.0001, P = 0.0001, and P < 0.0001, respectively). There was a significant inverse correlation between the serotonin cell density and the pain dimension of Birmingham IBS symptom questionnaire (r = -0.6, P = 0.0002). The density of PYY cells was 26.7 ± 1.6 cells/mm^2 in the controls, and 33.1 ± 1.4, 27.5 ± 1.4, 34.1 ± 2.5, and 41.7 ± 3.1 cells/mm^2 in the IBS-total, IBS-D, IBS-M, and IBS-C patients, respectively. This density differed significantly between patients with IBS-total and IBS-C and the controls (P = 0.03 and < 0.0001, respectively), but not between controls and, IBS-D, and IBS-M patients (P = 0.8, and P = 0.1, respectively). The density of PYY cells correlated significantly with the degree of constipation as recorded by the Birmingham IBS symptom questionnaire (r = 0.6, P = 0.0002). There were few PP-, enteroglucagon-, and somatostatin-immunoreactive cells in the biopsy material examined, which made it impossible to reliably quantify these cells.

CONCLUSION: The decrease of ileal serotonin cells is associated with the visceral hypersensitivity seen in all IBS subtypes. The increased density of PYY cells in IBS-C might contribute to the constipation experienced by these patients.

Key words: Computer image analysis; Irritable bowel syndrome; Ileum; Peptide YY; Serotonin

Core tip: The present study investigated for the first time the ileal endocrine cells in patients with irritable bowel syndrome (IBS). It included a relatively a large cohort of patients and comprising all the IBS subtypes, namely diarrhea (IBS-D), a mixture of both diarrhea and constipation (IBS-M) and constipation (IBS-C). It showed that the density of serotonin cells is reduced in patients with IBS, regardless of the subtype. On the other hand the density of peptide YY (PYY) cells in the ileum of IBS-D and IBS-M patients did not differ from that of controls, but was significantly elevated in those with IBS-C. It was concluded that the reduction of ileal serotonin cells may be connected to the visceral hypersensitivity seen in all IBS subtypes and that increase in the PYY cell density in IBS-C, would slow the intestinal transit and cause constipation.

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INTRODUCTION

Irritable bowel syndrome (IBS) is a common chronic gastrointestinal functional disorder that is characterized by abdominal discomfort or pain, altered bowel habits, and bloating/abdominal distension[1,2]. IBS reportedly has a prevalence of 5%-20% and an incidence of about 200 per 100000 of the adult population[2-9]. IBS reduces the quality of life considerably in IBS patients and is an economic burden to society for various reasons, including overconsumption of healthcare resources and increased sick leave[10]. However, IBS is not known to be associated with the development of serious disease or with increased mortality[11,12].

The diagnosis of IBS is based on the assessment of symptoms and detailed, accurate, and clinically useful definitions of the syndrome that have been elaborated by the working parties responsible for producing the latest Rome Ⅲ Criteria[13,14]. In addition to these criteria, warning symptoms (so-called red flags) such as age > 50 years, short history of symptoms, nocturnal symptoms, weight loss, rectal bleeding, anemia, and the presence of markers for inflammation or infections should be excluded. IBS patients are sub-grouped on the basis of differences in the predominant bowel symptoms into diarrhea (IBS-D), constipation (IBS-C), both diarrhea and constipation (IBS-M), and un-subtyped IBS in patients with insufficient abnormality of stool consistency to meet criteria for IBS-C, -D, or -M[13,14]. Because of the overlap of symptomology with celiac disease and microscopic colitis, some gastroenterologists (including the present authors) believe that these disorders should be excluded in addition to applying the Rome Criteria[15].

Abnormalities in the endocrine cells in the stomach, duodenum, colon, and rectum have been reported in patients with IBS[16-29], but the ileal endocrine cells have not been investigated previously. The endocrine cell types differ markedly between the distal and proximal small intestine, probably due to the quite different functions performed by these two parts of the intestine. The proximal small intestine contains serotonin, secretin, cholecystokinin (CCK), gastric inhibitory polypeptide (GIP), and somatostatin cells, while the ileum has the same endocrine cell types as in the large intestine, namely serotonin, peptide YY (PYY), pancreatic polypeptide (PP), enteroglucagon, and somatostatin cells[30].

A recent study observed endocrine cell depletion in the ileum of patients with sporadic IBS[31]. This depletion was detected by chromogranin A, which is a common marker for endocrine cells[32-34]. The aim of the present study was to clarify the affected endocrine cell types by examining various ileal endocrine cells in the same cohort of IBS patients investigated using chromogranin A.

MATERIALS AND METHODS

Patients and controls

Ninety-eight patients (77 females and 21 males; mean age 35 years, range 18-66 years) with IBS according to Rome Ⅲ Criteria were included in the study[13,14]. The IBS subtypes were distributed as follows: 35 patients with IBS-D, 31 patients with IBS-M, and 32 patients with IBS-C. Symptoms had been present in all of the patients for many years, and the onset of their IBS symptoms could not be associated with any events, in particular gastrointestinal or other infections. All patients underwent a complete physical examination and were investigated using the following blood tests: full blood count, electrolytes, inflammatory markers, liver tests, and thyroid function tests. They also underwent further gastroscopy with duodenal biopsies, which were used to exclude celiac disease. All of the patients had been tested previously (*i.e.*, before they were referred to us) for lactose intolerance and the presence of intestinal infectious agents including parasites in the stool; the results of all of these tests were negative.

For comparison, the control group comprised 38 subjects (26 females and 12 males; mean age 40 years, range 18-65 years) who had submitted to colonoscopy for the following reasons: gastrointestinal bleeding,

where the source of bleeding was identified as hemorrhoids (n = 24) or angiodysplasia (n = 3), and health worries resulting from a relative being diagnosed with colon carcinoma (n = 11).

The patients were asked to complete the: Birmingham IBS symptom questionnaire. The Birmingham IBS symptom score questionnaire is a disease specific score to measure the symptoms of patients with IBS. It has been developed to be suitable for self-completion and has been found to be acceptable to patients. Its dimensions have good reliability, external validity and sensitivity[35]. The questionnaire comprises 11 questions based on the frequency of IBS related symptoms. Each question has a standard response scale with symptoms all being measured on a 5-point Likert scale ranging from 0 ("none of the time") to 5 ("all of the time"). There are three underlying dimensions: pain (3 items), diarrhea (5 items) and constipation (3 items)[35].

The study was performed in accordance with the Declaration of Helsinki and was approved by the Regional Committee for Medical and Health Research Ethics West, Bergen, Norway. All subjects gave oral and written consent to participate.

Colonoscopy, histopathology, and immunohistochemistry

Colonoscopy was performed on both the patients and the controls, segmental biopsy specimens were taken from the colon and rectum, and four biopsy samples were taken from the ileum of each subject. The biopsy samples were fixed overnight in 4% buffered paraformaldehyde, embedded in paraffin, and cut into 5-m sections. The sections were stained with hematoxylin-eosin, and immunostained by the avidin-biotin complex (ABC) method using the Vectastain ABC kit (Vector Laboratories). The sections were hydrated and immersed in 0.01% hydrogen peroxide in PBS buffer (pH 7.4) for 10 min to inhibit endogenous peroxidase. After washing in buffer, the sections were treated with 1% bovine serum albumin for 30 min to block the nonspecific binding sites, and then incubated with the primary antiserum/antibody at room temperature for 2 h. The sections were then washed in PBS buffer and incubated with biotinylated swine anti-mouse (in the case of monoclonal antibodies) or anti-rabbit IgG (in the case of polyclonal antibodies) diluted 1:200 for 30 min at room temperature. After washing the slides in PBS buffer, the sections were incubated for 30 min with avidin-biotin-peroxidase complex diluted 1:100, and then immersed in 3,3'-diaminobenzidine (DAB) peroxidase substrate (Vector laboratories), followed by counterstaining in hematoxylin[18]. The following primary antisera/antibodies were used: monoclonal mouse anti-serotonin (Dako, code no. 5HT-209), polyclonal anti-porcine peptide PYY (Alpha-Dagnostica, code PYY 11A), polyclonal rabbit anti-synthetic-human PP (Diagnostic Biosystems, code No. 114), polyclonal rabbit anti-porcine glicentin/glucagon (Acris Antibodies, code BP508), and polyclonal rabbit anti-synthetic-human somatostatin (Dako, code no. A566); these antibodies were used at dilutions of 1:1500, 1:1000, 1:800, 1:400, and 1:200, respectively.

Computerized image analysis

Quantification of the endocrine cells was done as described previously[36,37]. Measurements were performed using Olympus cellSens imaging software (version 1.7) on a computer linked to an Olympus microscope type BX 43 with an Olympus camera (DP 26). The number of immunoreactive cells and the area of the epithelial cells were measured. The numbers of endocrine cells in each field were counted manually by pointing and clicking the computer mouse, and the areas of the epithelium containing these cells were drawn manually using the computer mouse. A × 40 objective was used, for which each frame (field) on the monitor represented a tissue area of 0.14 mm^2. Each individual and peptide hormone was measured in ten randomly chosen fields. Immunostained sections from the IBS patients and controls were coded and mixed, and measurements were made by the same person (El-Salhy M) who was blind to the identity of the sections. The data from the fields were tabulated, and the cell density of the epithelium (in cells/mm^2) was computed and statistically analyzed.

Statistical analysis

The gender difference between patients and controls was tested by Fisher's exact test, and the age difference was tested by the Mann-Whitney nonparametric test. Differences between controls, all IBS patients (IBS-total), IBS-D, IBS-M, and IBS-C patients were tested by the Kruskal-Wallis nonparametric test with Dunn's post-test. Correlation was done by Spearman nonparametric test. The data are presented as mean ± SE values, and differences with $P < 0.05$ were considered to be statistically significant.

RESULTS

Gender and age characteristics of patients and controls

The gender and age distributions did not differ significantly between the patients and the controls (P = 0.27 and P = 0.18, respectively). The total score of Birmingham IBS symptom questionnaire was 21 ± 0.8, and the three underlying dimensions: pain, diarrhea, and constipation were 7.2 ± 0.4, 6.6 ± 0.4, and 7.2 ± 0.4, respectively.

Endoscopy, histopathology, and immunohistochemistry

The colon and rectum of both the patients and the control subjects were macroscopically normal. The ileum was macroscopically normal in all except in one control subject and three patients, in whom lymphoid hyperplasia was observed; this condition is a common finding in young individuals and has no pathological relevance. The results of histopathological examinations of the ileum, colon, and rectum were normal in both the patients and the controls.

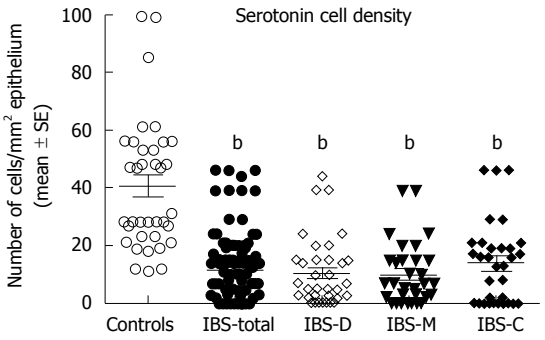

Figure 1 Serotonin cell densities in the ileum of controls and in all patients with irritable bowel syndrome, patients with diarrhea as the predominant symptom, patients with both diarrhea and constipation, and patients with constipation as the predominant symptom patients. $^{b}P < 0.01$ *vs* controls. IBS-total: All patients with irritable bowel syndrome; IBS-D: Patients with diarrhea as the predominant symptom; IBS-M: Patients with both diarrhea and constipation; IBS-C: Patients with constipation as the predominant symptom.

Serotonin-, PYY-, PP-, enteroglucagon-, and somatostatin-immunoreactive cells were found in the ileum of all of the subjects (*i.e.*, patients and controls), mostly in the crypts. These cells were basket- or flask-shaped.

Computerized image analysis

Serotonin cell density: The density of serotonin cells in the ileum was 40.6 ± 3.6 cells/mm^2 in the controls, and 11.5 ± 1.2, 10.7 ± 5.6, 10.0 ± 1.9, and 13.9 ± 1.4 cells/mm^2 in the IBS-total, IBS-D, IBS-M, and IBS-C patients, respectively. The serotonin cell density differed significantly between the controls and the IBS-total and IBS subgroups ($P < 0.0001$). Posttests showed that the density in the controls differed significantly from those in the IBS-total, IBS-D, IBS-M, and IBS-C groups ($P < 0.0001$, $P = 0.0001$, $P = 0.0001$, and $P < 0.0001$, respectively) (Figures 1 and 2). There was a significant inverse (negative) correlation between the serotonin cell density and the pain dimension of Birmingham IBS symptom questionnaire ($r = -0.6$, $P = 0.0002$). There was no significant correlation between the total score of Birmingham IBS symptom questionnaire, the diarrhea or constipation dimension ($r = -0.05$, $P = 0.8$; $r = -0.4$, $P = 0.8$; and $r = -0.2$, $P = 0.2$ respectively). The density of PYY cells was 26.7 ± 1.6 cells/mm^2 in the controls, and 33.1 ± 1.4, 27.5 ± 1.4, 34.1 ± 2.5, and 41.7 ± 3.1 cells/mm^2 in the IBS-total, IBS-D, IBS-M, and IBS-C patients, respectively. The PYY cell density differed significantly between the controls and the IBS-total and IBS subgroups ($P < 0.0001$). This density differed significantly between patients with IBS-total and IBS-C and the controls ($P = 0.03$ and < 0.0001, respectively), but not between controls and, IBS-D, and IBS-M patients ($P = 0.8$, and $P = 0.1$, respectively) (Figures 3 and 4). The density of PYY cells correlated significantly with the degree of constipation as recorded by the Birmingham IBS symptom questionnaire ($r = 0.6$, $P = 0.0002$). There was not significant correlation between the cell density of PYY and the total Birmingham IBS symptom questionnaire, the pain-, or diarrhea dimension ($r = 0.2$, $P = 0.2$; $r = 0.2$, $P = 0.06$; and $r = 0.1$, $P = 0.5$ respectively).

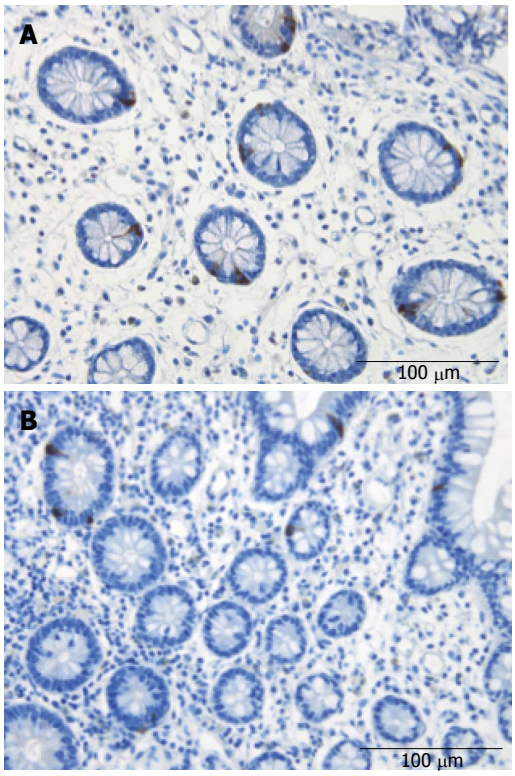

Figure 2 Ileal serotonin-immunoreactive cells in a control subject (A) and a patient with irritable bowel syndrome (B).

PP, enteroglucagon, and somatostatin cell densities: There were few PP-, enteroglucagon-, and somatostatin-immunoreactive cells in the biopsy material examined, which made it impossible to reliably quantify these cells.

DISCUSSION

The present study showed that the density of serotonin cells in the ileum is reduced in patients with sporadic (nonspecific) IBS, regardless of the subtype. This finding is similar to that reported in the colon of IBS patients[19]. On the other hand the density of PYY cells in the ileum of IBS-D and IBS-M patients did not differ from that of controls, but was significantly elevated in those with IBS-C. The observations made here on PYY cell density differ from those reported in the colon, where the density of PYY cells was reduced in IBS patients.

Abnormalities in the endocrine cells in the stomach, duodenum, colon, and rectum have been reported in patients with IBS[16-29]. The density of ghrelin cells in the stomach is lower in IBS-C and higher in IBS-D than in healthy controls[16]. In the duodenum, the cell densities of GIP and somatostatin are decreased in both IBS-D and IBS-C[18]. The densities of duodenal secretin and CCK cells are decreased in IBS-D but unchanged in IBS-C[18]. The duodenal serotonin cells are not affected in both IBS-D and IBS-C[18]. Postinfectious IBS was found to be associated with increased numbers of duodenal CCK cells but decreased numbers of serotonin cells[17]. Colonic serotonin and PYY cell densities have been found

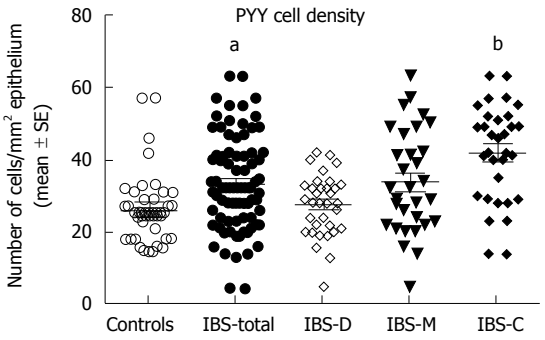

Figure 3 Peptide YY cell densities in the ileum of controls and in all patients with irritable bowel syndrome, patients with diarrhea as the predominant symptom, patients with both diarrhea and constipation, and patients with constipation as the predominant symptom patients. [a]*P* < 0.05, [b]*P* < 0.01 *vs* controls. PYY: Peptide YY; IBS-total: All patients with irritable bowel syndrome; IBS-D: Patients with diarrhea as the predominant symptom; IBS-M: Patients with both diarrhea and constipation; IBS-C: Patients with constipation as the predominant symptom.

to be low in both IBS-D and IBS-C[19]. In the rectum of patients with sporadic (nonspecific) IBS, the densities of PYY and enteroglucagon cells were significantly lower and that of somatostatin cells was significantly higher in both IBS-D and IBS-C than in the controls, whereas the serotonin cell density in these patients did not differ from that in healthy controls[21,38]. Rectal serotonin and PYY cell densities in postinfectious IBS have been reported to be elevated[23,25,27,39,40].

Serotonin cells are the predominant endocrine cell type in the ileum, which could account for the general reduction of ileal endocrine cells reported elsewhere[31]. Each intestinal crypt contains four to six pluripotent stem cells that differentiate through a series of cellular precursors (progenitors) into all epithelial cell types including enterocytes, goblet cells, Paneth cells, and endocrine cells[41-57]. It is possible that the reduction in ileal serotonin cells in IBS patients is due to abnormal cell differentiation from stem cell. This assumption gets support from the findings that depletions of endocrine cell in rejected ileum transplants are associated with marked depression in the expression of neurogenin-3 (NEUROG3) which is a early progenitor for endocrine cells and NeuroD, which is a transcription factor expressed by cells derived from NEUROG3[58]. Furthermore, a mutant NEUROG3 has been described in patients with congenital malabsorption diarrhea and lack of intestinal endocrine cells[59].

Serotonin activates the submucosal sensory branch of the enteric nervous system that conveys sensation from the gut to the central nervous system[60,61]. Serotonin modulates is known to modulate visceral sensitivity of the gastrointestinal tract[62,63]. It is therefore conceivable to conclude that the reduction of ileal serotonin cells may be connected to the visceral hypersensitivity seen in all IBS subtypes. It is difficult to establish whether the reduction in these ileal serotonin cells is primary or secondary to the visceral hypersensitivity. However, it is tempting to speculate that this abnormality is secondary to the visceral hypersensitivity and represents an adaptation mechanism

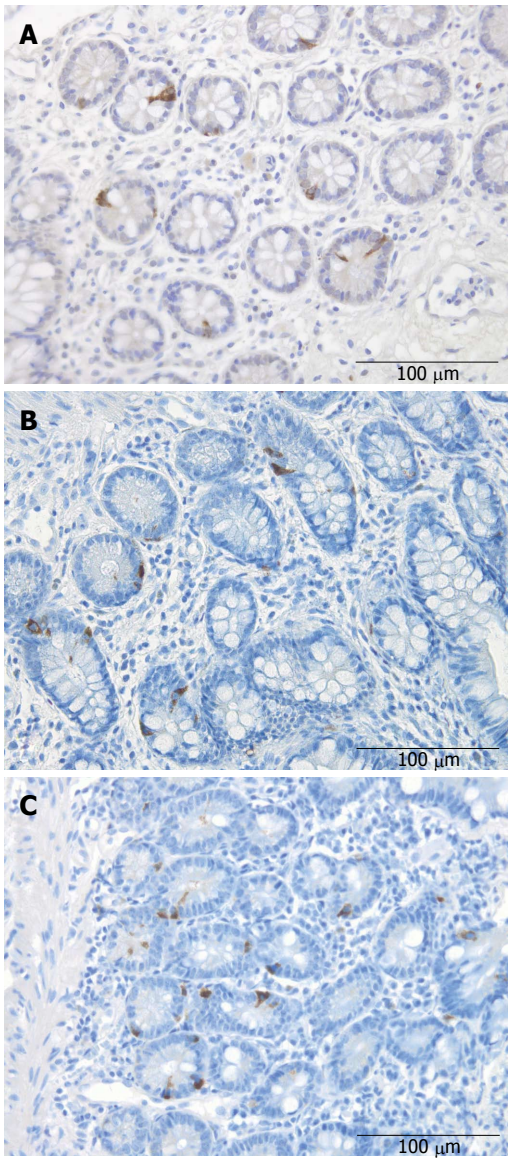

Figure 4 Peptide YY cells in the ileum of a control subject (A), a patient with irritable bowel syndrome into diarrhea (B), and a patient with irritable bowel syndrome into constipation (C).

for reducing the sensation conveyed from the gut to the central nervous system by serotonin. This assumption gets support from the present finding that serotonin cell density is correlated inversely to the pain score obtained by the Birmingham IBS symptom questionnaire. Speaking against this assumption are the findings that tryptophan hydroxylase (TPH)-1, which is the limiting enzyme for the synthesis in serotonin cells, and serotonin transporter (SERT) mRNA levels have been reported to be lower in the rectum and sigmoid colon of IBS patients than control subjects[64]. There was no difference, however, in the levels of (TPH)-1 and SERT between IBS patients with rectal hypersensitivity and those without[64].

PYY stimulates the absorption of water and electrolytes, and is a major regulator of the "ileal brake"[65-70]. Furthermore, PYY inhibits prostaglandin E2 and vasoactive intestinal polypeptide (VIP), which stimulate intes-

tinal fluid secretion[71-73]. Administration of PYY inhibits diarrhea in experimental mouse models by reducing intestinal fluid secretion and slowing colonic transit[74]. It is thus possible that the increase in the PYY cell density in IBS-C patients, and consequently the increase in PYY, would slow the intestinal transit by strengthening the ileal brake, increasing the absorption of water, and decreasing the secretion of the intestinal fluid in the distal small intestine *via* inhibiting VIP and prostaglandin E2. These effects would in turn cause constipation. In support of this conclusion is the observation that the density PYY cell correlated to the constipation score calculated from the Birmingham IBS symptom questionnaire.

The above summary indicates that most of the reported abnormalities in gastrointestinal endocrine cells are similar in all IBS subtypes[18,20,28-31]. However, in IBS-C the density of ghrelin cells in the oxyntic mucosa of the stomach was significantly lower than in healthy controls[16]. In addition to its role in regulating appetite and energy metabolism, ghrelin accelerates gastric and small- and large-intestinal motility[75-88]. It can be speculated that the low density of ghrelin cells previously reported and the high density of PYY cells observed in the present study explain why constipation predominates in the IBS-C subtype. On the other hand, in IBS-D patients the density of ghrelin cells in the stomach was significantly higher and the densities of duodenal secretin and CCK were lower than healthy volunteers[16,18]. Secretin inhibits gastric emptying and intestinal motility, and stimulates pancreatic bicarbonate and fluid secretions[30,89,90]. The secretion of pancreatic bicarbonate increases the pH of gut contents, which are highly acidic after leaving the stomach, and this is essential for lipid digestion as pancreatic lipase is irreversibly inactivated below pH 4.0[91]. CCK relaxes the proximal stomach in order to increase its reservoir capacity, inhibits gastric emptying and stimulates gall bladder contractions and pancreatic exocrine secretions of digestive enzymes from pancreatic exocrine glands[91-94]. The low densities of secretin and CCK cells in IBS-diarrhea patients could cause a rapid gastric emptying and acceleration of intestinal motility and ultimately diarrhea in these patients.

COMMENTS

Background

Irritable bowel syndrome (IBS) is a common chronic gastrointestinal disorder that is characterized by intermittent abdominal discomfort or pain, altered bowel habits, and bloating/abdominal distension. IBS reduces the quality of life considerably, but it is not known to be associated with the development of serious disease or with an increased mortality rates. Abnormal endocrine cells have been reported in the stomach, duodenum, colon, and rectum in patients with IBS, but the ileal endocrine cells have not been investigated previously.

Research frontiers

This study showed for the first time the ileal endocrine cells in patients with IBS are abnormal. It included a large cohort of patients and comprising all the IBS subtypes. It showed that the density of serotonin cells is reduced in patients with IBS, regardless of the subtype. Moreover, it revealed that the density of peptide Y (PYY) cells in the ileum of patients with IBS-D and IBS-, did not differ from that of controls, but was significantly elevated in IBS-C. It was concluded that the reduction of ileal serotonin cells may play a role in visceral hypersensitivity seen in IBS and that increase in the PYY cell density in IBS-C, may affect the intestinal transit and cause constipation.

Innovations and breakthroughs

The present study showed that the endocrine cells of the ileum of patients with IBS are abnormal. This together with the previously published results in the duodenum as well as in the stomach and large intestine of these patients indicate that the endocrine cells in all the segments of the gastrointestinal tract are affected. The gastrointestinal endocrine cells have specialized microvilli that project into the lumen and function as sensors for the luminal content and respond to luminal stimuli by releasing hormones into the lamina propria, which starts a chain reactions that progress throughout the entire neuroendocrine system. It is possible, therefore, that abnormalities the gut endocrine cells play a central role in the pathogenesis of IBS.

Applications

Identifying abnormalities in the gut endocrine cells may provide an effective tool in the treatment of IBS. Actually, a serotonin agonist is available in the market, which is approved for the treatment of chronic constipation.

Peer review

The study addresses the interesting areas of endocrine cells in IBS. The authors present a well conducted and written histologic study of enteroendocrine cell types in the ileum of IBS patients, correlating cell densities with symptoms and comparing them to control subjects.

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BRIEF ARTICLE

Serum 25-hydroxyvitamin D concentration and inflammatory bowel disease characteristics in Romania

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Abstract

AIM: To describe the relationship between vitamin D levels and inflammatory bowel disease (IBD) characteristics in northeastern Romanian patients.

METHODS: This was a prospective study of 47 consecutive IBD patients admitted to The Institute of Gastroenterology and Hepatology in Iasi, Romania between March 2011 and June 2012. The diagnosis of IBD was established based on endoscopic, histologic and radiologic findings. Demographic data, disease characteristics, ongoing treatments and biological parameters of patients (including markers of inflammation: C-reactive protein level, fibrinogen level, and erythrocyte sedimentation rate) were recorded. Serum vitamin D levels were measured and compared with age- and sex-matched healthy volunteers from the same geographic area. Vitamin D levels were defined as sufficient (> 30 ng/mL), insufficient (20-30 ng/mL), or severely deficient (< 20 ng/mL).

RESULTS: Thirty-three of the IBD patients included in this study had ulcerative colitis (UC) and 14 had Crohn's disease (CD). Only 24% of the UC patients and 21% of the CD patients had sufficient vitamin D levels. The vitamin D levels were significantly lower in the CD patients with moderate to severe disease activity compared to the CD patients in remission or with mild disease activity (16 ± 6 ng/mL *vs* 26 ± 7 ng/mL; 16 ± 6 ng/mL *vs* 31 ± 9 ng/mL, respectively, $P < 0.05$). Vitamin D levels in the UC patients were not influenced by disease activity and no correlation was observed with the inflammation markers tested (C-reactive protein, fibrinogen, and erythrocyte sedimentation rate). No association was observed between vitamin D levels and smoking status or ongoing medication (5ASA, steroids, and anti-TNFα). Newly diagnosed IBD patients had lower vitamin D levels than patients with established cases, though these differences were not significant (UC: 22 ± 9 ng/mL *vs* 26 ± 12 ng/mL; CD: 18 ± 6 ng/mL *vs* 27 ± 11 ng/mL, respectively). Although no association was found between the season during which the visit was scheduled and vitamin D levels, the UC patients assessed during the winter tended to have lower levels than those assessed during the summer (22 ± 9 ng/mL *vs* 28 ± 13 ng/mL, respectively).

CONCLUSION: Vitamin D levels are significantly reduced in IBD patients in northeastern Romania, with the lowest levels occurring in CD patients with moderate to severe disease activity.

Key words: Inflammatory bowel disease; Vitamin D level; Northeastern Romania; Crohn's disease activity; Seasonality

Core tip: This is the first prospective study assessing serum vitamin D levels in a Romanian population with

inflammatory bowel disease. The results of the study highlight the low prevalence of sufficient vitamin D levels in patients with Crohn's disease and ulcerative colitis. Furthermore, vitamin D levels were significantly lower in newly diagnosed cases, suggesting that disease treatment can help restore levels to some extent.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a debilitating condition characterized by a dysregulated mucosal immune response to intestinal microorganisms in a genetically susceptible host. While its pathogenesis is only partially understood, studies have shown that a complex combination of genetic, immune, environmental and microbial factors contribute to IBD. It is thought that IBD results from an inappropriate immune response to luminal antigens in the gut[1]. This disease is an important public health problem, affecting up to 0.5% of the population in developed countries and with increasing incidence in developing nations[2-4].

Recent studies describing the immunomodulatory function of vitamin D have suggested that vitamin D activity could play an extenuating role in the occurrence and progression of these autoimmune diseases[5-7]. Epidemiological studies suggest that the prevalence of vitamin D deficiency is high in patients with Crohn's disease (CD). Vitamin D deficiency is associated with a decreased exposure to sunlight, decreased oral vitamin D intake, malabsorption of vitamin D due to short gut syndrome or small bowel disease, bacterial overgrowth, and the use of cholestyramine for IBD symptom management[8]. Although patients with IBD are known to have an increased incidence of vitamin D deficiency, it remains unclear whether the deficiency contributes to and/or results from the disease. In this study, the vitamin D levels of northeastern Romanian IBD patients were compared with age- and sex-matched control subjects to assess the association between vitamin D levels and disease characteristics.

MATERIALS AND METHODS

Patient selection

This was a prospective study of outpatient and hospitalized IBD patients from the Institute of Gastroenterology and Hepatology Iasi in northeastern Romania between March 2011 and June 2012. The study was reviewed and approved by the local ethics review board. The diagnosis of IBD was established based on endoscopic, histologic and radiologic findings. Demographic data, disease characteristics, ongoing treatment, and biological parameters (including markers of inflammation, such as C-reactive protein level, fibrinogen level, and erythrocyte sedimentation rate) were recorded.

Disease characteristics

Disease location was classified according to the Montreal IBD classification system[9]. The disease activity for ulcerative colitis (UC) was determined using a total Mayo score[10], with a score of $\geqslant 3$ identifying a clinically active disease state (3-5, mild; 6-8, moderate; 9-12, severe). The Crohn's disease activity index was utilized to categorize CD activity[11], where clinically active disease was defined by a score $\geqslant 150$ (150-220, mild to moderate; 220-450, moderate to severe; > 450, fulminant to severe). Serum 25-hydroxyvitamin D concentrations were determined for all patients at the Bioclinica Laboratory using the high performance liquid chromatography method. Patients with confounding factors for serum vitamin D levels (*i.e.,* renal failure, liver disease, pregnancy and lactation, medications such as anticonvulsants and vitamin D supplements, and prominent malabsorption) were excluded. Ninety-four healthy age- and sex-matched volunteers from the same geographic area and without disorders of the gastrointestinal tract or bone, or other confounding factors served as control subjects. The vitamin D levels were compared across groups for effects related to sex, season, and IBD. CD and UC patients were analyzed separately for disease location, new/established disease, disease severity, and the impact of treatment on the vitamin D level. New disease (newly diagnosed) patients were those for whom the diagnosis of IBD was made just before enrolling the study, during that hospitalization, or within the previous four weeks. Established disease patients were those for whom the diagnosis of IBD was made more than four weeks prior. The relationship between markers of inflammation (*e.g.,* C-reactive protein level, fibrinogen level, and erythrocyte sedimentation rate), disease duration, smoking status, and vitamin D levels in all patients were analyzed.

Definition of vitamin D status

Although the definition of an acceptable 25-hydroxyvitamin D level is a matter of debate, a level greater than 30 ng/mL is considered optimal for maintaining a normal immune system. Therefore, we classified vitamin D levels as sufficient (> 30 ng/mL), insufficient (20-29 ng/mL), and deficient (< 20 ng/mL) for the purposes of this study.

Statistical analysis

All statistical analyses were performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, United States). The chi-square distribution test was performed for the majority of the analyses, and the Fisher's exact test was used in some cases when sample size was small. The threshold for statistical significance was set at $P < 0.05$, and data are

Table 1 Patient demographics *n* (%)

	Ulcerative colitis (*n* = 33)	Crohn's disease (*n* = 14)	Control subjects (*n* = 94)
Age, mean ± SD	42 ± 14	36 ± 9	42 ± 12
Sex, F/M	16/17	6/8	44/50
Disease duration in years, mean ± SD	3 ± 5	3 ± 3	
Newly diagnosed patient[1]	15 (45)	6 (43)	
Ulcerative colitis			
Proctitis	8 (24)		
Left colitis	12 (36)		
Pancolitis	13 (39)		
Crohn's disease			
Ileal		0 (0)	
Colonic		5 (36)	
Ileo-colonic		8 (57)	
Isolated upper digestive		1 (7)	
25-OH D in μg/L, mean ± SD	24 ± 11	23 ± 10	31 ± 13
Vitamin D deficiency	10 (30)	5 (36)	19 (20)
Vitamin D insufficiency	15 (45)	6 (43)	28 (30)
Vitamin D sufficiency	8 (24)	3 (21)	47 (50)
Season of clinical visit			
Winter	20 (61)	7 (50)	53 (56)
Summer	13 (39)	7 (50)	41 (44)

[1]Patients for whom the diagnosis of inflammatory bowel disease was established just prior to study inclusion.

reported as mean ± SD.

RESULTS

A total of 47 IBD patients and 94 healthy volunteers were included in this study. Thirty-three of the IBD patients had UC and 14 had CD. Demographic data and disease characteristics from these patients are presented in Table 1. Vitamin D levels were significantly lower in IBD patients compared to healthy controls (24 ± 10 ng/mL *vs* 31 ± 13 ng/mL, $P < 0.05$). However, IBD type did not influence this decrease, as no difference was found between the CD and UC patients. A significantly greater proportion of the male healthy controls had a sufficient vitamin D level than the male IBD patients (56% *vs* 25%, $P < 0.05$), but the difference between the female healthy controls and the female UC patients and respectively female CD patients did not reach the threshold of statistical significance (43% *vs* 24% *vs* 17%, $P = 0.16$). Additionally, while vitamin D levels in the healthy control group were significantly associated with the season ($P < 0.05$), this trend was not observed in the IBD patients. Although the vitamin D levels were lower in the winter compared to those during the summer in UC patients (22 ± 9 ng/mL *vs* 28 ± 13 ng/mL) and higher in CD patients (25 ± 8 ng/mL *vs* 21 ± 11 ng/mL, respectively), these differences were not significant.

In the UC patients, a lower serum vitamin D level was detected in a subgroup with extensive colitis (20 ± 7 ng/mL) compared to patients with left side colitis (30 ± 14 ng/mL); however, the difference was not significant. Similarly, a moderately reduced serum vitamin D level was observed in CD patients with involvement of only the colon compared to patients with additional involvement of the small bowel. Furthermore, newly diagnosed patients tended to have a lower vitamin D level than patients with established cases in the CD group (18 ± 6 ng/mL *vs* 27 ± 11 ng/mL) and the UC group (22 ± 9 ng/mL *vs* 26 ± 12 ng/mL). Although the severity of the flare in UC patients did not affect serum vitamin D levels, CD patients with moderate to severe disease activity had significantly lower vitamin D levels than patients in remission or with mild disease activity (16 ± 6 ng/mL *vs* 26 ± 7; 16 ± 6 ng/mL *vs* 31 ± 9 ng/mL, respectively, $P < 0.05$). Finally, there were no statistically significant associations between vitamin D levels and smoking or medication status, or with serum levels of C-reactive protein or fibrinogen, or with erythrocyte sedimentation rate.

DISCUSSION

The incidence and prevalence of IBD is higher in Northern Europe, North America, North Australia and New Zealand than in Asia[2,3]. Although the incidence of IBD in the Indian subcontinent is low, individuals migrating to developed countries in northern latitudes have an increased risk for developing the disease[12]. The association between vitamin D levels and sunlight exposure and IBD incidence is confounded by numerous factors, and therefore a causal relationship cannot be confirmed. The available reports on vitamin D levels in adults with IBD show a range of prevalence of vitamin D deficiency of 22% to 70% in CD cases and between 15% and 45% in those with UC[13-15]. This study found that only 24% of UC patients, 21% of CD patients, and 50% of healthy subjects were vitamin D sufficient. These results were somewhat unexpected, given that Romania is considered by some to be a sunny country, and therefore an adequate source of vitamin D.

Although low vitamin D concentrations have been reported in IBD[16,17], there are contradictory data regarding the correlation between 25-hydroxyvitamin D levels and IBD activity[18-20]. However, despite some reports showing no association[21], we anticipated that patients with small bowel CD would have lower vitamin D levels, as a study by Tajika *et al*[22] showed that 25-hydroxyvitamin D levels correlated with CD duration and activity. The current study indicates that, despite the high prevalence of vitamin D deficiency in IBD patients, serum vitamin D levels were only associated with IBD activity in CD patients, in contrast to a previous finding[19]. However, our results are in agreement with a similar study published by Lamb *et al*[23] showing that overall, vitamin D levels did not differ between patients with CD and patients with UC. Interestingly, our study indicated that newly diagnosed IBD patients tended to have lower vitamin D levels, though the small number of patients prohibited this difference from reaching statistical significance. It has been suggested that vitamin D deficiency in IBD patients is related to inadequate absorption[24]. Although this study demonstrated an increased incidence of vitamin D

deficiency in IBD patients, it is unclear whether the low vitamin D level is due to the IBD and associated inflammation of the gut, or if the IBD is a consequence of the immune disorders induced by the vitamin D deficiency.

A large population study found that high sunlight exposure was associated with a significantly decreased risk of CD[25]. Furthermore, patients with reduced sun exposure have lower serum 25-hydroxyvitamin D levels and increased disease activity[26]. While the onset and exacerbation of IBD is thought to show seasonal variation, suboptimal vitamin D levels have been observed even during the summer[27]. This study similarly failed to demonstrate a seasonality effect, though a trend for reduced deficiency rates in summer was observed in UC patients. In addition to sunlight exposure, steroid treatment for IBD may contribute to vitamin deficiency, as vitamin D-deficient patients are statistically more likely to be treated with steroids, but not other immunosuppressants such as infliximab, methotrexate, azathioprine, adalimumab or mercaptopurine[28], however, the sample size in this study was insufficient to allow for proper analysis of the impact of different treatments on vitamin D levels.

This is the first report of an assessment of vitamin D levels in IBD patients from northeastern Romania. Although the small number of patients was a limitation of the study, there are some advantages to the research design. Patients included in the study were excluded based on specific criteria, and disease activity was based on laboratory, clinical and endoscopic assessments. Furthermore, patients were compared with geographically similar control subjects, with all blood sampling performed in the same laboratory. The study highlights the low prevalence of sufficient vitamin D levels in IBD patients, and an association between vitamin D deficiency and moderate to severe CD. Future prospective cohort studies with larger patient samples are needed to determine the causal relationship between the vitamin D deficiency and the incidence of IBD. Moreover, the effect of vitamin D supplementation on IBD outcome should be investigated, which may provide insight to possible therapeutic or preventative measures for the treatment of IBD.

COMMENTS

Background

Inflammatory bowel diseases (IBDs) represent an important public health problem. Recent studies describing the immunomodulatory function of vitamin D suggest that it may influence the occurrence and progression of these autoimmune diseases.

Research frontiers

Although patients with IBD are known to have reduced vitamin D levels, it is unclear whether vitamin D deficiency contributes to or results from the disease. This study analyzes the association between vitamin D levels and disease characteristics in IBD patients from northeastern Romania.

Innovations and breakthroughs

This is the first study assessing vitamin D levels in a Romanian population. The study highlights the low prevalence of sufficient vitamin D level in IBD patients, as well the association between low vitamin D levels and moderate to severe Crohn's disease. The results are reinforced by strict exclusion criteria for patient enrollment, standardized clinical and endoscopic scoring systems for assessment of disease activity, and identical blood sampling and testing conditions for all patients and matched control subjects.

Applications

This report identifies a correlation with IBD severity and vitamin D deficiency. These results suggest that clinicians should assess the vitamin D status of their IBD patients and recommend vitamin supplementation to those with a deficiency.

Terminology

Vitamin D levels are assessed by serum 25-hydroxyvitamin D concentrations. Although the definition of an acceptable 25-hydroxyvitamin D level is a matter of debate, a level greater than 30 ng/mL is considered optimal for maintaining a normal immune system. This study categorized vitamin D levels as sufficient (> 30 ng/mL), insufficient (20-29 ng/mL), and deficient (< 20 ng/mL).

Peer review

This is the first prospective study assessing vitamin D levels in a Romanian population with IBD. The results indicate that IBD is associated with an increased incidence of vitamin D deficiency. Furthermore, patients with moderate to severe cases of Crohn's disease had significantly reduced vitamin D levels compared to patients in remission or with mild cases. These data suggest that vitamin D deficiency is an important factor in IBD, however more work is needed to determine whether the deficiency contributes to or results from the disease state.

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BRIEF ARTICLE

RAGE gene three polymorphisms with Crohn's disease susceptibility in Chinese Han population

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Abstract

AIM: To investigate the association of three polymorphisms in the receptor for advanced glycation end product (*RAGE*) gene with Crohn's disease (CD) risk in a Chinese population.

METHODS: A hospital-based case-control association study involving 312 CD patients and 479 healthy controls was conducted. Peripheral blood samples were collected from 791 study subjects, and genomic DNA was extracted. Genotyping was performed using polymerase chain reaction-ligase detection reaction method. The association between polymorphic genotype and CD predisposition was determined using odds ratio and 95% confidence interval (CI). Data were analyzed using Haplo.stats program.

RESULTS: Significant differences were observed between patients and controls in allele/genotype distributions of rs1800624 (P_{allele} = 0.012; $P_{genotype}$ = 0.005) and in allele distributions of rs2070600 (P = 0.02). The risk for CD associated with the rs1800624-A mutant allele decreased by 36% (95%CI: 0.47-0.88, P = 0.005) under the additive model and by 35% (95%CI: 0.46-0.91, P = 0.013) under the dominant model. Carriers of rs2070600-A mutant allele showed a 37% (95%CI: 1.02-1.83, P = 0.036) increased risk of developing CD relative to the GG genotype carriers. In haplotype analysis, haplotype T-A-G (in the order rs1800625, rs1800624, and rs2070600) decreased the odds of CD by 33% (95%CI: 0.49-0.94, P = 0.018).

CONCLUSION: CD is an immune-related disease with genetic predisposition. Genetic defects in the *RAGE* gene are strongly associated with CD in Chinese population.

Key words: Receptor for advanced glycation end product; Polymorphism; Crohn's diseases; Susceptibility; Association study

Core tip: The receptor for advanced glycation end products (RAGE) is a pattern recognition receptor involved in several pathophysiological processes associated with inflammation. Therefore, we considered that *RAGE* gene is a candidate gene susceptible to Crohn's disease (CD). This study is the first to investigate the association of the three most commonly studied polymorphisms in *RAGE* gene with CD risk in a Chinese population. The results suggest that *RAGE* rs1800624 and rs2070600 polymorphisms are associated with CD occurrence. The present findings support the hypothesis that a genetically impaired innate defense immunity system is a predisposing factor in the etiology of CD.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic relapsing inflammatory condition of the gastrointestinal tract that comprises two main subtypes, namely, Cohn's disease (CD) and ulcerative colitis (UC), which have overlapping but distinct clinical and pathological features. Considerable efforts have been devoted to elucidating the etiology and pathogenesis of IBD, but underlying regulatory and molecular mechanisms remain elusive. Epidemiological studies have documented that first-degree relatives of individuals with IBD have approximately 20-fold to 50-fold increased risk of developing the disease compared with the general population for CD and 10-fold to 20-fold increased risk for developing UC[1], suggesting a genetic basis for inheritance of IBD. Genome-wide linkage analyses have identified multiple candidate regions on several chromosomes for IBD. Meanwhile, numerous immunity-related genes have been found to locus on several IBD-susceptibility regions[2-4]. As a result of the importance of immunity in IBD, investigations on IBD-susceptibility genes involved in immunity have increasingly elicited research interest.

The receptor for advanced glycation end products (RAGE) is a member of the immunoglobulin protein family of cell surface molecules[5], and was initially isolated as the receptor of advanced glycation end products (AGEs) that accumulate during diabetes and senescence as a result of nonenzymatic glycation and oxidation of proteins and lipids[6]. RAGE has since been shown to bind a diverse set of ligands in addition to AGEs, including high-mobility group box-1, several members of the S100 protein family and b amyloid peptides, leading to the activation of several proinflammatory signaling pathways[7,8]. Presently, RAGE is a pattern recognition receptor involved in several pathophysiological processes associated with inflammation, such as diabetes complications[9], arthritis[10], systemic lupus erythematosus[11], multiple sclerosis[12], as well as CD[13]. In addition, animal model studies suggest that RAGE has an important function in innate defense mechanisms[14]. Meanwhile, recent studies have discovered nearly 20 naturally occurring RAGE-splicing variants in humans[15-19]. These isoforms are characterized by whole or parts of mRNA transcripts with missing or additional exons/introns or resulting from alternative splicing of the RAGE pre-mRNA and gene expression regulation[19]. Subsequent studies have presented convincing statistical evidence for a novel functional single nucleotide polymorphism, -374T/A, in RAGE being the CD susceptible locus in a German population, but not in an American population[13].

Considering that susceptibility genes in CD vary across different ethnic groups, we performed a hospital-based case-control association study on the three widely evaluated polymorphisms of *RAGE* gene and assessed the association between RAGE haplotypes and CD.

Table 1 Characteristics of Crohn's disease patients and healthy controls in a Chinese Han population

Characteristics	CD patients	Control subjects
Number	312	479
Male/female	205/107	308/171
Age, mean ± SD (yr)	34.0 ± 13.0	36.5 ± 15.1
Age at diagnosis (ys)		
< 17	42	63
17-40	212	324
> 40	58	92
CD behavior		
Inflammatory	179	
Stricturing	90	
Penetrating	43	
CD location		
Ileum	194	
Colon	27	
Ileocolon	91	
Perianal lesions		
Yes	92	
No	220	
Appendectomy		
Yes	18	
No	294	
Abdominal operation		
Yes	72	
No	240	

CD: Crohn's disease.

MATERIALS AND METHODS

Study populations

A hospital-based case-control study was conducted, involving 312 sporadic patients with CD and 479 healthy volunteers. All patients were recruited through the Outpatient Clinic at the Department of Gastroenterology at Ruijin Hospital, Shanghai Jiao Tong University School of Medicine as part of an ongoing project to examine genetic factors that contribute to the etiology of IBD. The demographics in the study population are summarized in Table 1. In this study, 42 patients were diagnosed under 17 years old in the CD group and there were 63 patients in the control group, without statistical difference in the proportion ($P = 0.97$).

Cases and controls were well matched by age and gender. Informed consent was obtained from all participants, and the study was approved by the Institutional Ethics Board of the Ruijin Hospital, Shanghai Jiao Tong University School of Medicine.

Genotyping

Blood samples (1 mL) were collected, and genomic DNA was extracted from white blood cells using TIANamp Blood DNA Kit (Tiangen Biotech Co., Ltd., Beijing, China). Genotyping was conducted by the PCR-ligase detection reactions (LDR) method using ABI 9600 system (Applied Biosystems, United States)[20]. The following cy-

Table 2 Genotype distributions and allele frequencies of the studied polymorphisms in patients and controls, and their risk prediction for Crohn's disease under three genetic models of inheritance

Polymorphism		Patients (n = 312)	Controls (n = 479)	P (χ^2)	Genetic models	OR; 95%CI
rs1800624	TT	248	343		Additive	0.64; 0.47-0.88
	AT	62	123	0.012	Dominant	0.65; 0.46-0.91
	AA	2	13		Recessive	0.23; 0.05-1.03
	A	10.6%	15.6%	0.005		
rs1800625	TT	228	353		Additive	0.98; 0.73-1.33
	CT	82	118	0.460	Dominant	1.03; 0.75-1.42
	CC	2	8		Recessive	0.38; 0.08-1.80
	C	13.8%	14.0%	0.940		
rs2070600	GG	174	303		Additive	1.29; 1.03-1.62
	AG	112	148	0.086	Dominant	1.37; 1.02-1.83
	AA	26	28		Recessive	1.46; 0.84-2.55
	A	26.3%	21.3%	0.022		

OR: Odds ratio.

cling parameters were used: 94 ℃ for 2 min; 35 cycles of 94 ℃ for 20 s; 56 ℃ for 20 s; 72 ℃ for 40 s; and a final extension step at 72 ℃ for 3 min. Two specific probes to discriminate the specific bases and one common probe were synthesized (available upon request). The common probe was labeled at the 3' end with 6-carboxy-fluorescein and phosphorylated at the 5' end. The following reaction conditions of LDR were followed: 94 ℃ for 2 min, 30 cycles of 94 ℃ for 30 s, and 56 ℃ for 3 min. After the reaction, 1 mL of LDR reaction products was mixed with 1 mL of ROX passive reference and 1 mL of loading buffer, denatured at 95 ℃ for 3 min, and chilled rapidly in ice water. The fluorescent products of LDR were differentiated using ABI sequencer 377 (Applied Biosystems, United States).

Statistical analysis

Comparisons between CD patients and controls were conducted using unpaired t test for continuous variables and χ^2 test for categorical variables. To avoid gross genotyping error, all polymorphisms were checked for consistency with Hardy-Weinberg equilibrium on a contingency table of observed-versus-predicted genotype frequencies using Pearson χ^2 test or Fisher's exact test. Genotypes were compared by logistic regression analysis under assumptions of additive, dominant, and recessive models of inheritance. $P < 0.05$ was considered statistically significant. Haplotype frequencies were estimated using the haplo.em program, and odds ratio (ORs) and 95% confidence interval (CI) were estimated using haplo.cc and haplo.glm programs according to a generalized linear model[21]. The haplo.score was used to model an individual's phenotype as a function of each inferred haplotype, which was weighted by their estimated probability to account for haplotype ambiguity. The haplo.em, haplo.glm, and haplo.score were evaluated using haplo.stats software (version 1.4.0) developed using R (http://www.r-project.org/). Study power was estimated using PS Power and Sample Size Calculations software (version 3.0).

RESULTS

Single-locus analysis

No deviations from Hardy-Weinberg equilibrium were found for all studied polymorphisms in the controls ($P > 0.05$). The genotype/allele distributions of the three selected polymorphisms in RAGE are shown in Table 2. Significant differences between CD patients and controls were observed in allele and genotype distributions of rs1800624 ($P_{allele} = 0.012$ and $P_{genotype} = 0.005$) and in allele distributions of rs2070600 ($P = 0.022$).

Notably, for rs1800624, the risk associated with mutant allele or genotype decreased by 36% (95%CI: 0.47-0.88) under the additive model and by 35% (95%CI: 0.46-0.91) under the dominant model. For rs2070600, a significant difference in association with CD under the additive (OR = 1.29; 95%CI: 1.03-1.62) and dominant (OR = 1.37; 95%CI: 1.02-1.83) models was observed. No significant association was detected for rs1800625 under the three genetic models.

Haplotype analysis

Haplotype frequencies of the three polymorphisms examined were estimated and compared between cases and controls (Table 3). The frequency of haplotype T-A-G (in the order rs1800625, rs1800624, and rs2070600) was significantly lower ($P = 0.005$) in patients, whereas the frequency of haplotype T-T-A was significantly higher ($P = 0.027$) in patients compared with the controls. After assigning the most common haplotype T-T-G as the reference, haplotype T-A-G decreased the odds of CD by 33% (95%CI: 0.49-0.94, $P = 0.018$).

Power calculation

Power calculation was performed to estimate the risk of obtaining false-negative results because of small sample size. Based on the two-sided test at the 0.05 significance level, this study exhibited 69% and 53% power in successfully detecting a significant association between rs1800624 and rs2070600 polymorphisms and risk of

Table 3 Haplotype frequencies of the studied polymorphisms in patients and controls, and their risk prediction for Crohn's disease

haplotype	Case	Control	Hapscore	*P* value	*P*sim	OR	95%CI	*P* value
T-T-G	49.40%	49.10%	0.075	0.94	0.92	Reference		
T-T-A	26.30%	21.30%	2.21	0.027	0.032	1.2	0.94-1.53	0.14
T-A-G	10.60%	15.60%	-2.82	0.005	0.002	0.67	0.49-0.94	0.018
C-T-G	13.80%	14.00%	-0.12	0.91	0.88	0.97	0.70-1.33	0.83

Alleles in haplotype were presented in order of polymorphisms *rs1800625*, *rs1800624* and *rs2070600*. OR: Odds ratio; P_{sim}: Simulated *P*.

CD under a dominant genetic model, respectively.

DISCUSSION

In this study, we investigated the role of three potential polymorphisms of *RAGE* gene in the risk of CD susceptibility in a Han Chinese population. The results reveal that the *RAGE* gene polymorphisms and the haplotype were associated with CD. Although the haplotype T-A-G (rs1800625- rs1800624- rs2070600), had low penetrance, it was negatively correlated with CD and could have a protective function in the latter. To the authors' knowledge, the present study is the first to explore the genetic susceptibility of *RAGE* gene to CD in Chinese population.

The rs1800624 polymorphism located at position -374 of the promoter region could be a functional polymorphism that results in reduced binding of a nuclear factor to a regulatory element of the *RAGE* gene promoter. *In vitro* experiments by Hudson *et al*[22] demonstrated that -374A resulted in a threefold increase in transcriptional activity compared with the T allele. Däbritz *et al*[13] found that *-374T/A RAGE* polymorphism is negatively associated with CD in a German population, supporting the hypothesis that the *-374T/A RAGE* polymorphism increases the levels of circulating soluble RAGE to neutralize proinflammatory mediators. However, this claim warrants further investigation.

Another variant (rs2070600, also known as *G82S* polymorphism) that causes a glycine-to-serine substitution at position 82 within the V-domain exhibits significantly different distribution between CD and control individuals (*P* allele = 0.022). The rs2070600 polymorphism is located in an exon in a region that has a crucial function in ligand binding. The 82S variant increases the ligand-binding affinity of the receptor[23,24], and consequently increases nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation and inflammatory gene expression. In addition, the *G82S* polymorphism is associated with reduced levels of soluble RAGE that magnifies the contribution from RAGE toward inflammation in a number of diseases[25]. The *G82S RAGE* polymorphism is associated with arthritis[23]. However, Däbritz *et al*[13] did not detect any association between G82S polymorphism and CD in either German or American population. They found that the minor A allele frequency of this polymorphism was below 5% in all study samples. In contrast, in the present study, the minor A allele frequencies were 26.3% and 21.3% in the cases and in the controls, respectively. The discrepancy in the results may be mainly explained by the heterogeneous genetic predispositions of individuals of different ethnicities. Genetic markers representing predisposition to IBD vary across geographical and racial groups. As proven by our previous meta-analyses, *CD14* gene *C-260T* polymorphism exhibited remarkable heterogeneity with UC across ethnic groups, with statistical significance in Asians but not in Caucasians[26]. However, considering the relatively smaller sample size in the present study, more studies are required to validate the effects for RAGE G82S.

In this study, haplotype analysis of the rs1800625-rs1800624-rs2070600 combination revealed four main and rare haplotypes. The common haplotype T-T-G showed a similar frequency between CD and control individuals (49.4% *vs* 49.1%). Compared with the common haplotype, haplotype T-A-G showed a highly significant negative correlation between CD and control individuals (10.6% *vs* 15.6%, *P* = 0.0049; OR = 0.67), suggesting that it is a protective haplotype. Haplotype analysis further confirmed the results that rs1800624 and rs2070600 are the susceptible loci for CD in the Chinese population.

However, this study has several drawbacks. First, the sample size is relatively small and may not produce efficient statistical power to detect a small genetic effect, resulting in a fluctuated estimation. Second, limited polymorphisms of *RAGE* gene associated with susceptibility to CD were shown, and other unidentified polymorphisms that influence the development of CD still remain to be discovered. Therefore, further related studies of a large sample size from different ethnic origins and biological studies should be carried out to verify this association. Third, data on circulating soluble RAGE levels are unavailable, so RAGE levels cannot be compared across genotypes.

In conclusion, this study revealed that polymorphisms and haplotypes of the *RAGE* gene are significantly associated with susceptibility to CD in the Chinese population. Moreover, this study leaves open the question of divergent genetic profiles across different ethnic groups. This study provides supporting evidence for further investigation on pathophysiological mechanisms of *RAGE* genes in CD.

COMMENTS

Background

The incidence of Crohn's disease (CD) is rising in China, although the exact etiology of CD remains elusive. Genome-wide linkage analyses and association

studies have identified multiple candidate genes susceptible to CD. However, the susceptibility genes in CD may vary across different ethnic groups.

Research frontiers

The receptor for advanced glycation end products (RAGE) is a pattern recognition receptor involved in several pathophysiological processes associated with inflammation, including *CD-374T/A* polymorphism in RAGE which was found to be associated with CD in a German population, but not in the American population. In the present study, the authors demonstrated that RAGE rs1800624 and rs2070600 polymorphisms were associated with CD occurrence in a Chinese population.

Innovations and breakthroughs

Studies that investigate the association between *RAGE* gene polymorphisms and CD susceptibility risk are limited. This study is the first to investigate the association between the three most commonly studied polymorphisms in *RAGE* gene and CD risk in a Chinese population.

Applications

The present findings further support a genetically impaired innate defense immunity system as one predisposing factor in the etiology of CD, which is a prerequisite for development of new treatment strategies for CD.

Terminology

RAGE is a member of the immunoglobulin protein family of cell surface molecules that binds multiple structurally diverse ligands, leading to the activation of several proinflammatory signaling pathways. RAGE is a pattern recognition receptor involved in several pathophysiological processes associated with inflammation, and has important functions in innate defense mechanisms.

Peer review

This research is a well-designed case-control study demonstrating that RAGE rs1800624 and rs2070600 polymorphisms are associated with CD occurrence in a Chinese population. This study adds evidence for a genetically impaired innate defense immunity system as one predisposing factor in the etiology of CD.

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BRIEF ARTICLE

Prognostic value of M30/M65 for outcome of hepatitis B virus-related acute-on-chronic liver failure

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Abstract

AIM: To determine the prognostic value of circulating indicators of cell death in acute-on-chronic liver failure (ACLF) patients with chronic hepatitis B virus (HBV) infection as the single etiology.

METHODS: Full length and caspase cleaved cytokeratin 18 (detected as M65 and M30 antigens) represent circulating indicators of necrosis and apoptosis. M65 and M30 were identified by enzyme-linked immunosorbent assay in 169 subjects including healthy controls (n = 33), patients with chronic hepatitis B (CHB, n = 55) and patients with ACLF (n = 81). According to the 3-mo survival period, ACLF patients were defined as having spontaneous recovery (n = 33) and non-spontaneous recovery which included deceased patients and those who required liver transplantation (n = 48).

RESULTS: Both biomarker levels significantly increased gradually as liver disease progressed (for M65: P < 0.001 for all; for M30: control *vs* CHB, P = 0.072; others: P < 0.001 for all). In contrast, the M30/M65 ratio was significantly higher in controls compared with CHB patients (P = 0.010) or ACLF patients (P < 0.001). In addition, the area under receiver operating characteristic curve (AUC) analysis demonstrated that both biomarkers had diagnostic value (AUC ⩾ 0.80) in identifying ACLF from CHB patients. Interestingly, it is worth noting that the M30/M65 ratio was significantly different between spontaneous and non-spontaneous recovery in ACLF patients (P = 0.032). The prognostic value of the M30/M65 ratio was compared with the Model for End-Stage Liver Disease (MELD) and Child-Pugh scores at the 3-mo survival period, the AUC of the M30/M65 ratio was 0.66 with a sensitivity of 52.9% and the highest specificity of 92.6% (MELD:AUC = 0.71; sensitivity, 79.4%; specificity, 63.0%; Child-Pugh: AUC = 0.77; sensitivity, 61.8%; specificity, 88.9%).

CONCLUSION: M65 and M30 are strongly associated with liver disease severity. The M30/M65 ratio may be a potential prognostic marker for spontaneous recovery in patients with HBV-related ACLF.

Key words: Acute-on-chronic liver failure; Chronic hepatitis B virus infection; Liver disease stage; Liver disease severity; Serum M65 level; Serum M30 level; Prognostic value

Core tip: Massive hepatic cell death is a key characteristic of liver failure. Enzyme-linked immunosorbent assay was used to measure M65 and M30 in a chronic hepatitis B (CHB) infection cohort which included healthy controls, CHB and acute-on-chronic liver failure (ACLF) patients. Elevated M65 and M30 differentiated CHB or ACLF patients from healthy controls and gradually increased with disease severity. M30/M65 was significantly increased in ACLF patients with spontaneous recovery (P = 0.032), and the AUC of this ratio at the 3-mo survival period was 0.661 (sensitivity: 52.9%) with a high specificity (92.6%) compared with the Model for End-Stage Liver Disease and Child-Pugh scores.

Zheng SJ, Liu S, Liu M, McCrae MA, Li JF, Han YP, Xu CH, Ren F, Chen Y, Duan ZP. Prognostic value of M30/M65 for outcome of hepatitis B virus-related acute-on-chronic liver failure. *World J Gastroenterol* 2014; 20(9): 2403-2411 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i9/2403.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i9.2403

INTRODUCTION

Chronic hepatitis B virus (HBV) infection is one of the leading causes of liver-associated death and disease in China[1,2], and accounts for more than 83% of overall liver failure and over 90% of acute-on-chronic liver failure (ACLF) cases[3-5]. Recently, ACLF has been found to be an increasingly recognized entity encompassing acute deterioration of liver function in patients with chronic liver disease worldwide[6-8]. Prompt and accurate prediction of outcome and correct medical decision making can improve outcome in ACLF patients. The currently used prognostic models, such as the Model for End-Stage Liver Disease (MELD) and Child-Pugh scores, were not specifically designed for patients with HBV-related ACLF, therefore, it is worth exploring new potential biomarkers which might improve the prognostic model for HBV-related ACLF patients.

Dysregulation of hepatocyte apoptosis plays an important role in liver disorders[9]. M30-antigen, the caspase-cleaved cytokeratin-18, is used to detect apoptosis[10-13]. Uncleaved cytokeratin-18, detectable as M65-antigen[14], is also released from dying cells (due to both apoptosis and necrosis).

An elevation in M30 was identified in patients with liver disorders, including chronic hepatitis C (CHC)[15-17]. A previous study reported that elevated serum M30 was positively associated with a higher degree of fibrosis in CHC patients, and it was suggested that elevated M30 may be more sensitive than aminotransferase for identifying liver injury, particularly in CHC patients with normal aminotransferase profiles[18]. Another study showed that the measurement of serum M30 or M65 can accurately differentiate nonalcoholic steatohepatitis (NASH) or simple steatosis from controls, with M65 being a better diagnostic indicator than M30[19]. In all of these reports, an elevation in serum M30 showed some correlation with disease severity in the early stage of liver injury. In addition, M30 showed clinical significance as a potential prognostic marker in end-stage liver disorders, however, these findings are still controversial. Rutherford *et al*[20] reported that the median levels of tumor necrosis factor-alpha and M30 were at least 10-fold greater in acute liver failure (ALF) than in either CHC or normal controls. Based on the serum level of M30 and commonly measured clinical variables, the same group developed a prognostic index for ALF which was validated in 250 patients, and found that M30 and clinical variables at study entry most accurately identified patients who would require liver transplantation (LT) or would die[21]. In contrast, Volkmann *et al*[22] found that patients who spontaneously recovered from ALF had a significantly higher level of serum M30 than patients who required LT or who died. Several additional reports have evaluated the role of overall cell death in NASH, hepatitis C virus infection and liver failure[23-25], and controversies still exist regarding which mode of cell death predominates in various disease stages[9].

However, as potential prognostic biomarkers for clinical outcome of ACLF, an investigation of M30 and M65 in patients with HBV infection as the single etiology has not been reported. In this study, we first investigated the profile of cell death at different disease stages, and in order to determine if there was a correlation with liver damage, we then investigated the association between M30 and M65 with alanine aminotransferase (ALT) or aspartate aminotransferase (AST) in cohort patients. Finally, we evaluated these cell death biomarkers as predictive markers of outcome in ACLF patients at the 3 mo-survival period, and further compared these biomarkers with the MELD and Child-Pugh scores.

MATERIALS AND METHODS

Study participants

From July 2008 to October 2011, 136 subjects including patients with CHB (n = 55), patients with ACLF (n = 81), and 33 healthy individuals were enrolled. All subjects underwent a physical examination, biochemical screening, a blood coagulation test and a liver function test.

All patients had a history of chronic HBV infection and were either hepatitis B surface antigen (HBsAg) or HBV DNA positive (detected by real-time polymerase chain reaction assay) for more than 6 mo before enrollment. Patients with CHB had persistent ALT elevation or had repeated elevation of ALT and/or inflammation as seen on histological examination of liver biopsy

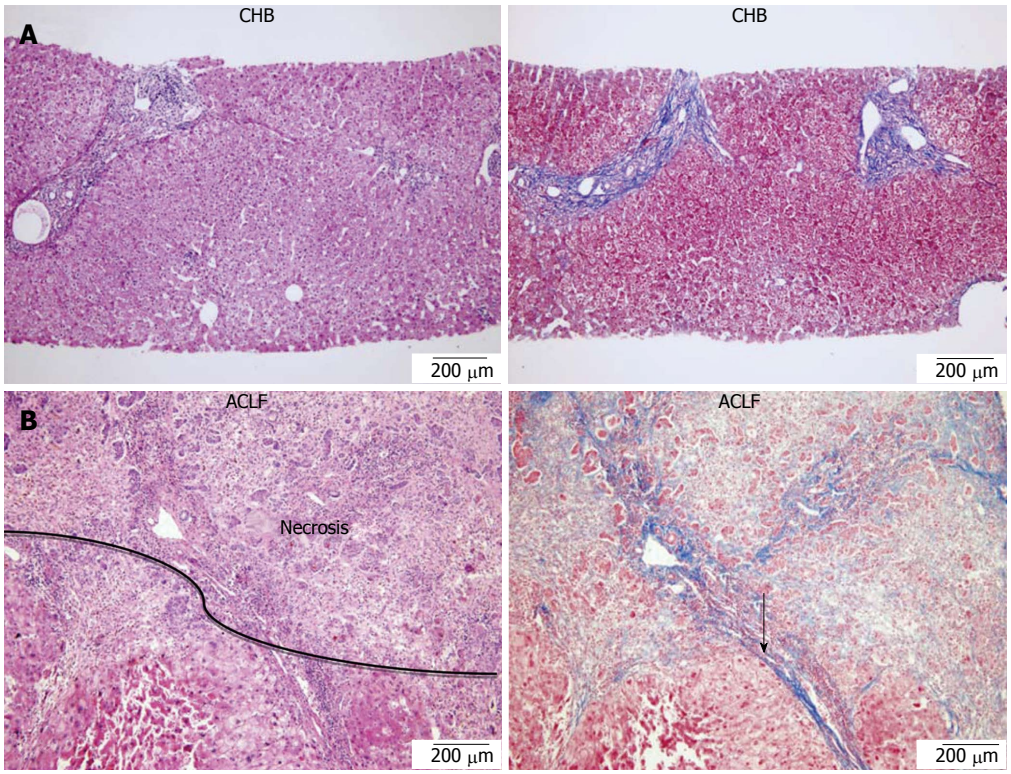


Figure 1 Histology of tissue sample for chronic hepatitis B and and acute-on-chronic liver failure in representative patients. A: Chronic hepatitis B (CHB, mild): Mild enlarged portal tract is infiltrated by lymphocytes without interface hepatitis. Spot necrosis is in the lobule (left, HE, × 100). Portal tract shows mild fibrosis (right, Masson trichrome); B: Acute-on-chronic liver failure (ACLF): Massive necrosis of parenchyma with cirrhotic nodule remaining (left, HE, × 100). Cirrhotic nodule is surrounded by fibrous tissue (arrow) (right, Masson trichrome).

samples[26]. Patients with ACLF fulfilled the following criteria: presented with evidence of acute hepatic insult manifesting as jaundice and coagulopathy, complicated quickly by ascites and/or encephalopathy and with previously diagnosed or undiagnosed chronic liver disease. In addition, serum total bilirubin was ≥ 171 μmol/L, prothrombin time increased significantly and the percentage of prothrombin activity (PTA) was ≤ 40%[6]. Thirty-six of 55 CHB patients underwent liver biopsy, and a pathological analysis was conducted. The liver tissues from 19 ACLF patients who underwent LT were also analyzed by the same experienced pathologist (Figure 1).

All patients with ACLF were followed for at least 3 mo to determine the 3-mo survival period. The patients who died or underwent LT during the admission period were recorded, and patients who were discharged before the end of the follow-up period were monitored *via* telephone. The spontaneous recovery group was defined as patients with ACLF who survived for more than 3 mo, whereas the non-spontaneous recovery group was defined as patients with ACLF who died within 3 mo or received a liver transplant during this time. The 3-mo start point was set as the day when the serum was collected.

Patients presenting with other viral hepatitis, hemochromatosis, Wilson's disease, autoimmune hepatitis, primary biliary cirrhosis, sclerosing cholangitis, biliary obstruction, alpha-1 antitrypsin deficiency, or malignancies were excluded from the study cohort.

Peripheral fasting blood samples were collected from patients on the morning of the second day after hospitalization, centrifuged at 1000 *g* for 15 min at 4 ℃ and the serum collected. Aliquoted serum was then immediately stored at -80 ℃ until used in the enzyme-linked immunosorbent assay (ELISA) analysis.

The study protocol was reviewed and approved by the Institutional Review Board of Beijing Youan Hospital, Capital Medical University, Beijing, China. Written informed consent was obtained from each participant before initiation of the study. The study was carried out according to the Declaration of Helsinki and the guidelines of the International Conference on Harmonization for Good Clinical Practice.

Detection of serum M65 and M30

All serum samples collected were first blinded and then tested in duplicate. Total cytokeratin 18 and caspase-cleaved cytokeratin 18 fragments were measured using the M65 and M30 Apoptosense ELISA kits purchased from PEVIVA (Bromma, Sweden) according to the manufacturer's recommended procedure[27]. In brief, serum samples were first placed into 96-well plates which had been coated with a mouse monoclonal antibody as a catcher. Following incubation for 4 h at room temperature, the plate was washed five times with phosphate buffered saline. A horseradish peroxidase conjugated antibody (against either M65 or M30) was then used to de-

Table 1 Demographics and clinical characteristics of study subjects

	Health control (n = 33)	CHB (n = 55)	ACLF (n = 81)	P value
Male	26 (78.79)	46 (83.64)	68 (83.95)	0.788
Age (yr)	39.73 ± 7.12	38.62 ± 12.08	42.84 ± 11.12	0.075
HBV DNA viral load (Log)	-	6.66 ± 1.49	5.76 ± 1.73	0.000
ALT (U/L)	18.71 ± 9.33	212.07 ± 290.77	527.06 ± 790.14	< 0.0001
AST (U/L)	21.70 ± 11.84	109.85 ± 158.32	401.72 ± 571.31	< 0.0001
Total bilirubin (μmol/L)	11.07 ± 3.47	28.43 ± 30.72	394.98 ± 200.16	< 0.0001
Albumin (g/L)	46.17 ± 2.05	40.02 ± 3.73	31.06 ± 3.99	< 0.0001
Crea (μmol/L)	66.72 ± 16.14	72.10 ± 12.66	80.96 ± 50.26	0.1768
International normalized ratio	-	1.03 ± 0.11	2.40 ± 1.00	< 0.0001
Prothrombin activity	-	98.86% ± 17.34%	31.34% ± 11.24%	< 0.0001
White blood cell (× 10^9/L)	5.85 ± 1.22	5.23 ± 1.30	8.19 ± 3.79	< 0.0001
Platelet (× 10^{12}/L)	223.55 ± 22.09	170.72 ± 56.45	105.90 ± 58.13	< 0.0001

Data are presented as numbers (percentage) or mean ± SD. CHB: Chronic hepatitis B; ACLF: Acute-on-chronic liver failure; HBV: Hepatitis B virus; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

tect the presence and concentration of M65 or M30. The absorbance at 450 nm was determined in a microplate reader within 30 min of setting up the assay.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 for Windows, and the results are expressed as mean ± SD unless otherwise stated. The continuous variables among multigroups were analyzed by one way ANOVA or the Kruskal-Wallis test depending on data distribution, and pairwise comparisons between groups were performed using the LSD-t or Mann-Whitney test. In addition, the independent-samples t test or Mann-Whitney test was employed to compare the spontaneous recovery group with the non-spontaneous recovery group in ACLF patients. Categoric variables were analyzed by the χ^2 test. Correlation analysis was performed by Spearman's correlation. The diagnostic value of the index with significant difference was assessed by the area under the receiver operating characteristic (ROC) curves. A two-sided P value < 0.05 was considered statistically significant.

RESULTS

Patients

In this patient population, 84% of patients were male (114/136) and 16% were female (22/136), and the age range was 19-65 years with an average age of 41.13 ± 11.66 years. In ACLF patients, 16.05% were female (13/81) and 83.95% were male (68/81) with an average age of 42.84 ± 11.12 years. Previous studies repeatedly reported that male Chinese patients developed HBV-related ACLF more frequently than female ones[3-5]. Therefore, in the present study, gender-matched healthy individuals and CHB patients were enrolled. The demographic and clinical characteristics of the subjects are summarized in Table 1.

In ACLF patients, approximately 40% (33/81) fulfilled the criteria for spontaneous recovery, and 59% (48/81) either required LT (n = 19) or died (n = 29) and fulfilled the criteria for non-spontaneous recovery. The clinical features of ACLF patients with spontaneous recovery or non-spontaneous recovery are summarized in Table 2.

Serum M65 and M30 levels can identify different disease stages

Using ELISA, we detected M30 and M65 levels in patient sera and found that both M65 and M30 levels significantly increased gradually as liver disease progressed (for M65: P < 0.001 for all between different groups; for M30: control *vs* CHB, P = 0.072; others: P < 0.001 for all). The median M65 level was 458.40 (range: 270.80-958.40) U/L in healthy controls, 876.60 (range: 268.20-4000.00) U/L in CHB patients and 1980.55 (range: 770.80-8020.00) U/L in ACLF patients, and for M30, the median level was 218.80 (range: 168.76-1234.40) U/L in healthy controls, 248.40 (range: 146.88-2000.00) U/L in CHB patients and 537.60 (range: 295.32-3000.00) U/L in ACLF patients (Figure 2A and B). Therefore, our analysis revealed that disease severity was associated with more hepatocyte necrosis and apoptosis.

Hepatocyte necrosis (M65) plays a stronger role than apoptosis (M30) in liver disease deterioration

Measurement of the M30/M65 ratio by ELISA has been reported to be useful in reflecting the balance between hepatocyte necrosis and apoptosis in early stage liver disorders[19,23]. However, few reports on the late stage of liver disorders are available. Therefore, we analyzed the M30/M65 ratio in our cohort. Compared with the control group [0.54 (range: 0.23-3.49)], the median M30/M65 ratio gradually decreased in the CHB group [0.46 (range: 0.20-0.73)] and was lowest in the ACLF group [0.33 (range: 0.15-0.95)], and the differences were statistically significant between the healthy controls and each disease stage (P < 0.05, respectively). The differences between healthy controls and CHB patients and between ACLF patients and CHB patients were statistically significant (P = 0.01, 0.003) (Figure 2C). These results demonstrated that more hepatocyte necrosis than apoptosis occurred during the development of liver disease deterioration.

Table 2 Clinical features of acute-on-chronic liver failure patients based on different outcomes

Features	Spontaneous recovery (n = 33)	Non spontaneous recovery (n = 48)	P value
Age (yr)	41.06 ± 12.48	44.06 ± 10.04	0.235
Male	29 (87.88)	38 (79.17)	0.308
HBV DNA viral load (Log)	5.78 ± 1.62	5.29 ± 2.31	0.487
ALT (U/L)	540.98 ± 555.99	525.15 ± 920.58	0.098
AST (U/L)	412.38 ± 525.00	397.69 ± 605.02	0.645
Total bilirubin (μmol/L)	371.38 ± 199.37	407.99 ± 211.36	0.435
Albumin (g/L)	31.67 ± 3.58	30.82 ± 4.24	0.492
Crea (μmol/L)	68.20 ± 31.59	88.34 ± 59.38	0.094
Prothrombin activity	35.89% ± 10.62%	28.45% ± 10.73%	0.003
International normalized ratio	2.04 ± 0.49	2.67 ± 1.18	0.003
White blood cell ($\times 10^9$/L)	8.40 ± 3.66	8.20 ± 4.10	0.571
Platelet ($\times 10^{12}$/L)	119.44 ± 51.17	95.60 ± 61.65	0.017
M30 (U/L)	891.35 ± 741.27	695.24 ± 385.15	0.663
M65 (U/L)	2477.23 ± 1671.10	2497.77 ± 1503.84	0.488
M30/M65	0.38 ± 0.13	0.32 ± 0.10	0.032
MELD score	23.07 ± 4.89	27.59 ± 6.55	0.002
Child-Pugh score	11.42 ± 1.23	12.49 ± 1.14	0.000

Data are presented as numbers (percentage) or mean ± SD. HBV: Hepatitis B virus; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; MELD: Model for End-Stage Liver Disease.

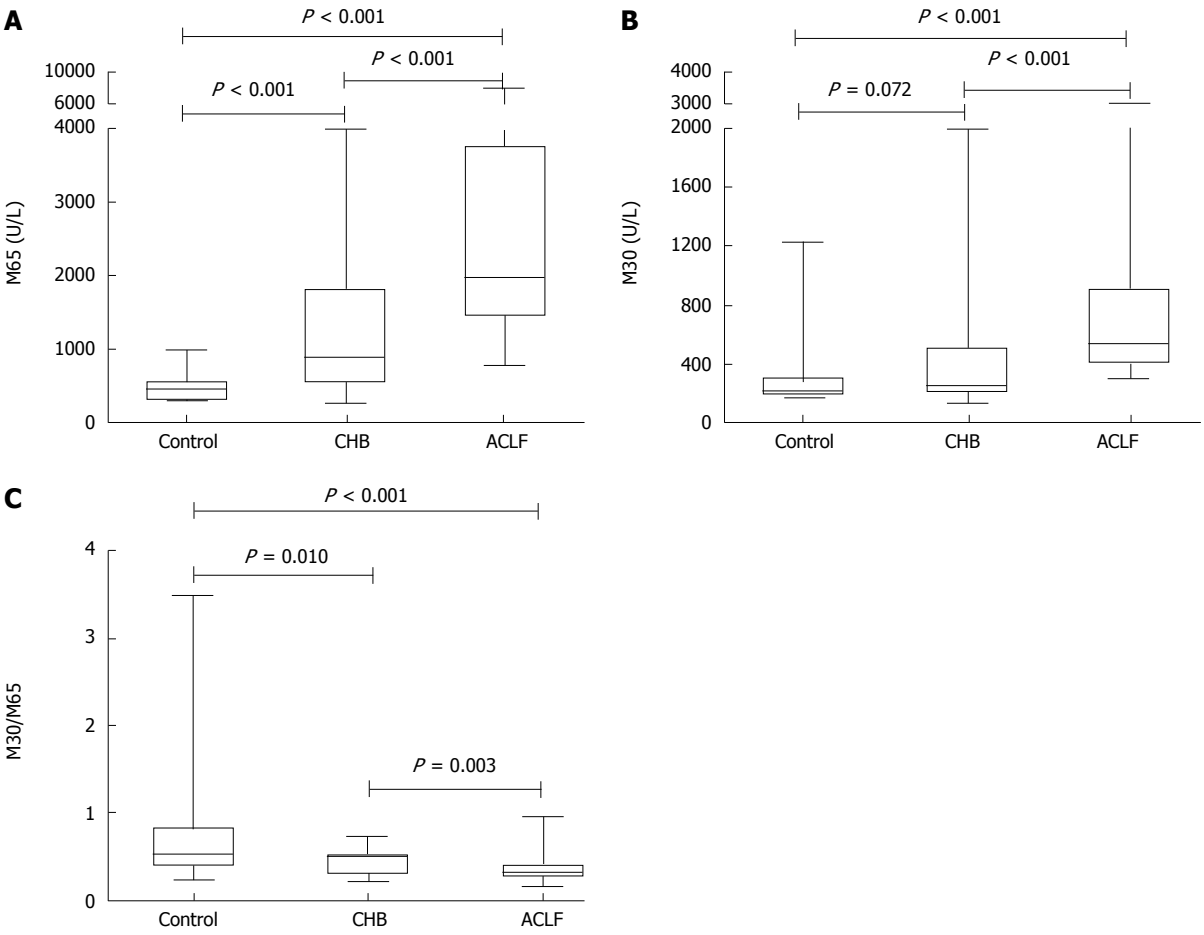
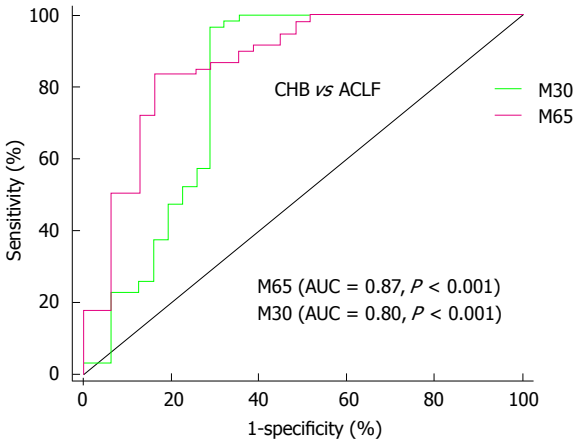


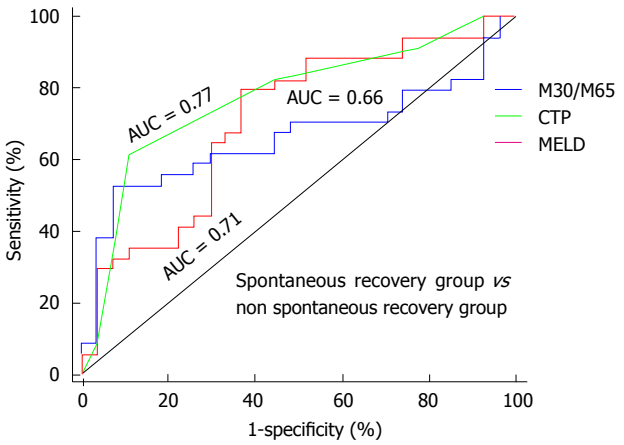
Figure 2 Serum M65, M30 levels and M30/M65 ratio can significantly identify healthy control, chronic hepatitis B and acute-on-chronic liver failure. A: Serum M65 levels can significantly indentify healthy control, chronic hepatitis B (CHB) and acute-on-chronic liver failure (ACLF); B: Serum M30 levels can significantly indentify healthy control, CHB and ACLF; C: The ratio of M30/M65 can significantly identify healthy control, CHB and ACLF. The length of the box represents the interquartile range. The line through the middle of the box is the median. The whiskers indicate the minimum and maximum values.

In order to confirm overall hepatic cell death levels in the corresponding liver tissues, representative liver tissues from CHB and ACLF patients were analyzed pathologically. It was observed that the severity of liver cell necro-



Variables	Se	Sp	PPV	NPV	Cut off (U/L)
M65	0.836	0.839	0.812	0.784	1260.30
M30	0.967	0.710	0.868	0.917	333.35

Figure 3 Both serum M65 and M30 levels show diagnostic performance to identify acute-on-chronic liver failure patients in cohort patients. Se: Sensitivity; Sp: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; CHB: Chronic hepatitis B; ACLF: Acute-on-chronic liver failure; AUC: Area under the curve.



Variables	Se	Sp	PPV	NPV	Cut off (U/L)
M30/M65	0.529	0.926	0.900	0.610	0.300
MELD	0.794	0.630	0.735	0.625	23.540
CTP	0.618	0.889	0.848	0.596	12.50

Figure 4 Comparison of the prognostic values of Model for End-Stage Liver Disease, Child-Pugh score and the ratio of M30/M65 in acute-on-chronic liver failure patients. Se: Sensitivity; Sp: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; MELD: Model for End-Stage Liver Disease; AUC: Area under the curve.

sis increased from CHB to ACLF, and massive necrosis of hepatic parenchyma was observed in ACLF (Figure 1).

Significant association between serum levels of M65 and M30 and ALT, AST in cohort patients

To date, the most convenient and economic indicator of liver damage is the measurement of serum ALT[28]. Interestingly, in the present study, there was also a strong positive correlation between M65 level and ALT and AST in ACLF patients (for ALT: $r = 0.43$; for AST: $r = 0.42$, $P = 0.000$ for all) and CHB patients (for ALT: $r = 0.53$; for AST: $r = 0.64$, $P = 0.000$ for all).

Similar to M65, M30 levels were significantly correlated with ALT and AST, in both ACLF patients (for ALT: $r = 0.34$, $P = 0.007$; for AST: $r = 0.32$, $P = 0.013$) and CHB patients (for ALT: $r = 0.64$, $P = 0.000$; for AST: $r = 0.63$, $P = 0.000$).

In aggregated patients including both CHB and ACLF, as expected, strong and significant positive correlations were observed between M65 or M30 and ALT (M65: $r = 0.54$, $P = 0.000$; M30: $r = 0.53$, $P = 0.000$), and AST (M65: $r = 0.64$, $r = 0.55$, both $P = 0.000$). These results revealed that both serum M65 and M30 had diagnostic value in reflecting liver injury.

Analysis of serum M65 and M30 levels to identify ACLF patients

Good diagnostic indicators able to identify patients suffering from end-stage liver diseases who are at high risk of dying are essential for good clinical management. The ROC analysis showed that both serum M65 and M30 levels had significant diagnostic value for identifying ACLF patients [M65: cutoff: 1260.30 U/L, AUC = 0.87 (95%CI: 0.78-0.95); sensitivity, 83.6%; specificity, 83.9%; M30: cutoff: 333.35 U/L, AUC= 0.80 (95%CI: 0.68-0.92); sensitivity, 96.7%; specificity, 71.0%) (Figure 3).

In order to investigate the possible prognostic value of cell death biomarkers, based on 3-mo survival time in ACLF patients, they were defined as the spontaneous recovery group and the non-spontaneous recovery group. We investigated whether there were possible associations between M65 or M30 and outcome, however, we did not find significant differences between them. We then analyzed a possible association between the M30/M65 ratio and outcome, and, for the first time, a significant difference was found between the M30/M65 ratio and the 3-mo survival period ($P = 0.032$) in patients with HBV-related ACLF. These data are shown in Table 2.

We then conducted an AUC analysis to compare the prognostic performance of the M30/M65 ratio, MELD and Child-Pugh scores. Although the AUC of the M30/M65 ratio was not as high as those of the MELD and Child-Pugh scores, the AUC (95%CI) of the M30/M65 ratio (cut off value: 0.3) was 0.66 (0.52-0.80) with a sensitivity of 52.9% and the highest specificity of 92.6% compared with the MELD and Child-Pugh scores [MELD: AUC = 0.71 (95%CI: 0.58-0.84); sensitivity, 79.4%; specificity, 63.0%; Child-Pugh: AUC = 0.77 (95%CI: 0.65-0.89); sensitivity, 61.8%; specificity, 88.9%) (Figure 4).

DISCUSSION

A fuller understanding of the mechanism(s) underpinning the different disease stages leading to better judgment of liver disease severity, coupled with additional biomarker-guided individualized therapeutic strategies, is crucial for the improved management of patients with end-stage liver disease. Currently available biomarkers, such as aminotransferase levels, PTA and bilirubin, are representative indicators of the outcome of liver dam-

age. Hepatocyte death plays a fundamental role in disease staging and severity, and consequently the identification of new biomarkers reflecting basic hepatocyte necrosis or apoptosis could provide useful insights into disease development and clinical outcomes. In this context, the measurement of serum M65 and M30 levels has allowed the investigation of both overall cell death and analysis of the cell death profile at different disease severities resulting from chronic HBV infection, and evaluation of the potential prognostic value of these biomarkers in ACLF patients.

This is the first study carried out in a Chinese cohort with a single etiology (chronic HBV infection, a major disease in China) to demonstrate that elevated M65 and M30 levels can differentiate CHB or ACLF patients from healthy controls. In addition, the gradual increase in the levels of M65 and M30 and the associated progression through disease staging allowed a correlation between these biomarkers and ALT or AST to be established. Furthermore, ROC-area under curve (ROC-AUC) analysis demonstrated that both biomarkers showed diagnostic value (AUC $\geqslant$ 0.80) in identifying ACLF patients from CHB patients. Interestingly, it is worth noting that there was a significant difference in the M30/M65 ratio between ACLF patients with spontaneous recovery and those with non-spontaneous recovery which included those who required LT or who died (P = 0.032) at the 3-mo survival period. The prognostic value of the M30/M65 ratio was compared to the MELD and Child-Pugh scores at the 3-mo survival period, and the AUC for the M30/M65 ratio was 0.66 with the highest specificity of 92.6%. Our study demonstrated that the M30/M65 ratio may be a potential prognostic marker for clinical outcome in ACLF patients.

Measurement of serum ALT is widely accepted as the most sensitive indicator of hepatic injury. The ultimate outcome of hepatic damage is increased hepatic cell death (necrosis and apoptosis), thus, in this study M65 and M30 levels were found to be strongly correlated with ALT and AST in the whole patient group and in each subgroup. Similar results have been reported previously in earlier stages of liver disorders including steatosis, CHC, and acute liver failure[18,20,29].

Disease-associated cell death typically follows one of two patterns, apoptosis or necrosis[30]. The majority of earlier studies in patients with NASH, hepatitis C virus infection, liver failure[22-24], and acetaminophen hepatotoxicity[31] focused primarily on apoptosis, and as a result, there is some controversy regarding which mode of cell death predominates in the various forms of liver disease, and the severity of overall liver damage that results from each[9]. The present results have shown, for the first time in chronic HBV infection, that although both patterns of cell death are important at different stages of liver disease, hepatocyte apoptosis predominates at earlier stages of disease. As the disease stage progresses there is a gradual switch to necrosis, and eventually necrosis predominates in the late stage of liver disease. The ratio of apoptosis *vs* necrosis found in control samples was 54% *vs* 46%. However, in CHB this ratio changed to 46% *vs* 54%, and was 33% *vs* 67% in ACLF. Our investigation revealed that hepatic cell necrosis has a stronger role than apoptosis in the progression of chronic HBV infection. Although at present it is unclear which specific factors contribute to the switch, this phenomenon was observed and its clinical significance should be addressed.

We then analyzed the diagnostic value of M65 and M30 in identifying ACLF patients, as expected the ROC analysis showed that the AUC for both biomarkers reached 0.867 (AUC for M65: 0.87; M30: 0.80) in identifying ACLF patients in the patient cohort.

A potential association between hepatic cell death biomarkers and the 3-mo survival period in ACLF patients was further evaluated. Neither M65 nor M30 was significantly different between the spontaneous recovery group and the non-spontaneous recovery group during the 3-mo survival period; however, a decrease in the ratio of M30/M65 was significantly associated with poor prognosis. In order to demonstrate the possible prognostic performance of the M30/M65 ratio, we analyzed and compared its prognostic power with that of the MELD and Child-Pugh score. Although the prognostic performance of the M30/M65 ratio was not as good as that of the MELD or Child-Pugh score, it still showed some prognostic power with the highest specificity of 92.6% (MELD: 63.0%; Child-Pugh: 88.9%). This is the first report on HBV-related ACLF to show that the M30/M65 ratio has prognostic value for predicting the clinical outcome of ACLF patients at the 3-mo survival period.

It is widely accepted that the development of liver failure is complicated and that multiple organs are involved. In HBV-related ACLF, the pathogenesis is more complex, and we consider it impossible that any single parameter can provide sufficient prognostic value. Only a model with a combination of several major and fundamental clinical parameters can have the power to provide more comprehensive and accurate prognostic values for the clinical outcome of liver failure, such as the classic MELD or Child-Pugh scores. Serum M65 has been evaluated and served as a major parameter in a modified MELD score for predicting spontaneous recovery in ALF[21,32].

However, evidence on the predictive value of cell death biomarkers was not consistent for liver disorders in previous reports. In an ALF cohort study, neither apoptotic nor necrotic cell death markers accurately predicted survival in ALF patients[33]. However, conflicting positive associations have also been reported in ALF patients[20-22]. Most previous reports revealed that either M30 or M65 had prognostic value, however, our investigation demonstrated that only the M30/M65 ratio has prognostic value and not each biomarker alone. We consider that the M30/M65 ratio represents the overall hepatic cell death profile, not like each cell death biomarker which reflects either cell necrosis or apoptosis. However, further well-designed studies with larger cohorts are needed to re-evaluate these possible predictive values.

In addition to the above findings, a previous study

found that the M30/M65 ratio was significantly decreased in congestive heart failure-induced ALF compared with HBV-related ALF[32]. However, the number of patients with HBV as the single cause of ALF was limited in this study[32]. Thus, a study on the cell death profile in liver failure induced by different etiologies may provide important clinical results. To date, our investigation is the first to include 81 ACLF patients with chronic HBV infection as the single cause.

This study has some limitations. Our study demonstrated, for the first time, that the M30/M65 ratio showed prognostic value in ACLF patients at the 3-mo survival period, however, further research will be required to combine this ratio into the classic model or establish a new and more accurate prognostic model. The present study was a single center investigation; the findings need to be confirmed in larger multi-center studies.

In summary, this study confirmed, for the first time in a Chinese cohort with a single etiology (chronic HBV infection), that a decrease in the M30/M65 ratio is associated with poorer clinical outcomes and that the M30/M65 ratio has prognostic significance for ACLF patients at the 3-mo survival period.

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COMMENTS

Background

In China, over 90% of acute-on-chronic liver failure (ACLF) patients have chronic hepatitis B virus (HBV) infection. Prompt and accurate prediction of prognosis can improve the outcome of HBV-ACLF patients. To date, there are no prognostic models specifically designed for HBV-ACLF patients. Massive hepatic cell death is a fundamental characteristic of liver failure. Using enzyme-linked immunosorbent assay (ELISA) to measure serum M65 and M30, hepatocyte apoptosis and necrosis can be determined. Although a previous study demonstrated that M30 or M65 had prognostic value in patients with acute liver failure due to various etiologies, the prognostic value of M30 and M65 in patients with HBV-ACLF has not been reported.

Research frontiers

Some studies have demonstrated that serum levels of M30 or M65 are associated with liver disease severity. Although numerous studies found that serum M30 or M65 showed clinical significance as potential prognostic markers in acute liver failure, these studies had a limited sample size and conflicting results, and no reports in patients with HBV-related ACLF are available.

Innovations and breakthroughs

The measurement of circulating indicators of cell death can reflect hepatocyte apoptosis and necrosis. A previous study reported that an elevation in serum M30 or M65 was correlated with liver disease severity. Although M30 or M65 showed clinical significance as potential prognostic markers in acute liver failure due to various etiologies, these findings are controversial. In order to study their prognostic value in patients with chronic HBV infection as the single etiology, M30 and M65 were identified by ELISA in 169 subjects including healthy controls (n = 33), chronic hepatitis B (CHB) patients (n = 55) and ACLF patients (n = 81). Both biomarker levels significantly increased gradually as liver disease progressed. In contrast, the M30/M65 ratio was significantly higher in controls compared with CHB or ACLF patients. receiver operating characteristic-area under the curve (ROC-AUC) analysis demonstrated that both biomarkers had diagnostic value (AUC $\geq$ 0.80) in identifying ACLF patients from CHB patients. Moreover, a significant difference in the M30/M65 ratio in ACLF patients was found at the 3-mo survival period. The prognostic value of the M30/M65 ratio was compared to the MELD and Child-Pugh scores at the 3-mo survival period, and its AUC was 0.66 with the highest specificity of 92.6%.

Applications

The present study demonstrated that both M30 and M65 were strongly associated with liver disease severity. The M30/M65 ratio may be a potential prognostic marker for spontaneous recovery in patients with HBV-related ACLF.

Terminology

M65: The M65 ELISA detects a common epitope present in the full-length cytokeratin 18 as well as in the caspase-cleaved fragment and is released from dying cells (due to both apoptosis and necrosis). M30: The M30 detection antibody recognizes a neo-epitope mapped to positions 387 to 396 of cytokeratin 18, which is only revealed after caspase cleavage of the protein and is considered a selective biomarker of apoptosis.

Peer review

This is the first report in HBV related ACLF that M30/M65 ratio has prognostic value for predicting ACLF patient clinical outcomes at 3-mo survival period. The results are interesting and suggest that serum M65 and M30 are strongly associated with liver disease severity. M30/M65 ratio might be a potential prognostic marker for spontaneous recovery in HBV-related ACLF patients.

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META-ANALYSIS

Addition of prokinetics to PPI therapy in gastroesophageal reflux disease: A meta-analysis

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Abstract

AIM: To investigate the efficacy of adding prokinetics to proton pump inhibitors (PPIs) for the treatment of gastroesophageal reflux disease (GERD).

METHODS: PubMed, Cochrane Library, and Web of Knowledge databases (prior to October 2013) were systematically searched for randomized controlled trials (RCTs) that compared therapeutic efficacy of PPI alone (single therapy) or PPI plus prokinetics (combined therapy) for GERD. The primary outcome of those selected trials was complete or partial relief of non-erosive reflux disease symptoms or mucosal healing in erosive reflux esophagitis. Using the test of heterogeneity, we established a fixed or random effects model where the risk ratio was the primary readout for measuring efficacy.

RESULTS: Twelve RCTs including 2403 patients in total were enrolled in this study. Combined therapy was not associated with significant relief of symptoms or alterations in endoscopic response relative to single therapy (95%CI: 1.0-1.2, P = 0.05; 95%CI: 0.66-2.61, P = 0.44). However, combined therapy was associated with a greater symptom score change (95%CI: 2.14-3.02, P < 0.00001). Although there was a reduction in the number of reflux episodes in GERD [95%CI: -5.96-(-1.78), P = 0.0003] with the combined therapy, there was no significant effect on acid exposure time (95%CI: -0.37-0.60, P = 0.65). The proportion of patients with adverse effects undergoing combined therapy was significantly higher than for PPI therapy alone (95%CI: 1.06-1.36, P = 0.005) when the difference between 5-HT receptor agonist and PPI combined therapy and single therapy (95%CI: 0.84-1.39, P = 0.53) was excluded.

CONCLUSION: Combined therapy may partially improve patient quality of life, but has no significant effect on symptom or endoscopic response of GERD.

Key words: Gastroesophageal reflux diseases; Proton pump inhibitors; Prokinetics; GABA-B receptor agonists; Treatment; Meta-analysis

Core tip: Proton pump inhibitors (PPIs) are generally accepted as the standard treatment of care for gastroesophageal reflux disease (GERD). However, many patients undergoing PPI treatment have no effective symptomatic relief. Although many studies have shown the clinical efficacy of adding prokinetics to PPI therapy in GERD, others have shown no therapeutic benefit. The efficacy and safety of combined prokinetic and PPI therapy for GERD remain controversial. In this retrospective meta-analysis, we find no advantage for the addition of prokinetics to a PPI therapeutic regimen, relative to PPI alone. However, combination therapy may improve symptom score and patient quality of life.

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INTRODUCTION

Gastroesophageal reflux disease (GERD) is a common condition affecting 10%-20% of Europeans[1] and 3%-7% of Asians[2]. Based on an endoscopy study, the prevalence of erosive reflux esophagitis (RE), a chronic form of GERD associated with damage to the esophagus, ranges from 6% to 10% in Asia[3]. Since RE is more likely to be detected by endoscopy than non-erosive reflux disease (NERD), the incidence of RE is higher than that of NERD. Symptoms of GERD, which include heartburn, non-cardiac chest pain, acid regurgitation, chronic cough, bloating and belching, may seriously affect quality of life of some patients. Furthermore, GERD is linked with serious complications, such as hemorrhage, peptic stricture, Barrett's esophagus, and esophageal adenocarcinoma[3-5]. Both NERD and RE are subtypes of GERD. NERD presents clinically with acid reflux and heartburn with no mucosal break, whereas RE patients have mucosal damage detectable by endoscopy[6]. The mechanisms underlying GERD may include esophageal hypersensitivity and transient lower esophageal sphincter relaxation (TLESR)[7]. Studies show that changes in diet, physical activity, and BMI increase the risk for GERD[3]. NERD may be due to visceral hypersensitivity, prolonged contraction of the lower esophagus, and other psychological factors[8].

Proton pump inhibitors (PPIs) are generally accepted as the standard treatment paradigm for GERD. Although many patients with RE have symptomatic relief with this drug alone[9], many patients have no symptomatic resolution[10-13]. Overall, 30% of GERD patients, 10%-15% of RE patients, and 40%-50% of NERD patients do not experience symptom alleviation with conventional PPI therapy[14-16]. New PPI formulations and regenerative types of acid-suppressive drugs for GERD are urgently needed.

Prokinetics are agents that increase lower esophageal sphincter pressure (LESP), enhance esophageal peristalsis, and augment gastric emptying. These include 5-hydroxytryptamine (5-HT) receptor agonists, GABA-B receptor agonists, dopamine receptor antagonists, and others. Five-HT receptor agonists increase acetylcholine release from parasympathetic nerve roots and promote gastric emptying and bowel motility[14,17], and are frequently used in combination with PPI therapy. Cisapride is a canonical prokinetic agent with equal efficacy as a 5-HT4 receptor agonist and a H2 histamine receptor antagonist. In addition to protecting the esophageal mucosa, it was reported that cisapride increased LEST and esophageal peristaltic amplitude; however, cisapride is now prohibited in Europe due to its detrimental side effects on the cardiac system[18]. Mosapride, another 5-HT4 agonist, is a structural analog of cisapride with less cardiac side effects[19,20]. It has been approved in Asia for the treatment of some functional gastrointestinal disorders, such as functional dyspepsia. Baclofen and lesogaberan (AZD 3355) were developed as selective GABA-B agonists based on their inhibition of TLESR and reflux episodes[21]. A phase Ⅱ study reported that lesogaberan combined with PPI modestly improved GERD symptoms[22], but its efficacy and safety were not determined.

Although many studies have shown that addition of a prokinetic to PPI can improve the symptoms of GERD, there is still some controversy in the literature. The efficacy and safety profiles of combination prokinetics and PPI therapy regimens relative to PPI monotherapy for GERD remain unclear. Here, we performed a retrospective meta-analysis to identify the efficacy and safety of these two types of treatments in GERD.

MATERIALS AND METHODS

Literature search

All eligible articles in English published prior to October 2013 were searched from PubMed, Cochrane Library, and Web of Knowledge. The search strategy consisted of a combination of the following MESH terms and text words: gastroesophageal reflux diseases, GERD, non-erosive reflux diseases, NERD, reflux esophagitis, RE, proton pump inhibitors, PPI, prokinetics, and GABA-B receptor agonists. A Cochrane filter for identifying randomized controlled trials (RCTs) was applied to the search results, and all potentially relevant abstracts and citations were retrieved for further review. Furthermore, we searched the bibliographies of selected trials obtained through the electronic screen to identify additional studies of interest.

Criteria for inclusion

Articles were eligible for inclusion in this meta-analysis if they met the following criteria: (1) Participants were diagnosed with GERD (RE or NERD); (2) Participants were 18 years or older; (3) Patients receiving PPI monotherapy were compared with patients receiving combined prokinetic and PPI therapy; (4) The study was a RCT; (5) Criteria for successful treatment were clearly defined; and (6) Treatment lasted for two or more weeks.

Criteria for exclusion

Publications were excluded according to the following criteria: (1) Studies comparing H2 receptor antagonist plus prokinetic to H2 receptor antagonist; (2) Participants with complications in addition to GERD; and (3) Missing or unclear data for final outcomes of interest.

Data extraction

To avoid bias in the data abstraction process, two investigators (Ren LH and Chen WX) independently abstracted

Table 1 Characteristics of the 12 randomized controlled trials included in this meta-analysis of the effects of combined prokinetic and proton pump inhibitor therapy in gastroesophageal reflux diseases *n* (%)

Ref.	Country	Participants (*n*)	Duration of study	Female	Age (yr)	BMI (kg/m^2)
Vakil *et al*[23], 2013	United States	460	6 wk	254 (55.2)	44	28
Cho *et al*[24], 2013	South Korea	50	4 wk	26 (52.0)	46	21
Shaheen *et al*[28], 2013	United States	661	4 wk	376 (56.9)	48	28
Ndraha *et al*[29], 2011	Indonesia	60	2 wk	40 (66.7)	42	24
Hsu *et al*[2], 2010	Taiwan	96	8 wk	48 (50.0)	47	24
Boeckxstaens *et al*[22], 2011	United States	244	4 wk	82 (33.6)	50	27
Miwa *et al*[12], 2011	Japan	200	4 wk	120 (60.0)	52	22
Beaumont *et al*[27], 2009	United States	16	2 wk	8 (50.0)	54	Not reported
Madan *et al*[19], 2004	India	68	8 wk	23 (33.8)	35	Not reported
Smythe *et al*[30], 2003	United Kingdom	23	4 wk	3 (13.0)	62	Not reported
van Rensburg *et al*[25], 2001	United Kingdom	350	8 wk	213 (60.9)	47	28
Vigneri *et al*[26], 1995	Italy	175	12 mo	58 (33.1)	45	Not reported

Table 2 Symptom response in ten studies

Ref.	Intervention	Combined therapy, improved/treated	Single therapy, improved/treated
Cho *et al*[24], 2013	Esomeprazole 40 mg/d + mosapride 30 mg *tid*	19/24	13/19
Hsu *et al*[2], 2010	Lansoprazole 30 mg/d + mosapride 5 mg *tid*	39/44	41/50
Madan *et al*[19], 2004	Pantoprazole 40 mg *bid* + mosapride 5 mg *tid*	25/28	23/33
Miwa *et al*[12], 2011	Omeprazole 10 mg/d+ mosapride 5 mg *tid*	45/97	42/95
van Rensburg *et al*[25], 2001	Pantoprazole 40 mg/d + cisapride 20 mg *bid*	120/173	129/177
Vigneri *et al*[26], 1995	Omeprazole 40 mg/d + cisapride 10 mg *tid*	31/35	28/35
Beaumont *et al*[27], 2009	PPI + baclofen 20 mg *tid*	4/12	6/12
Boeckxstaens *et al*[22], 2011	PPI + lesogaberan 65 mg *bid*	21/104	11/105
Shaheen *et al*[28], 2013	PPI + lesogaberan 60/120/180/240 mg *bd*	110/458	22/122
Vakil *et al*[23], 2013	PPI + baclofen 20/40/60 mg *qd*	110/240	21/54

PPIs: Proton pump inhibitors.

the data, recorded the first author, year of study, study design, and study population characteristics, and compared the results. All data were checked by a third reviewer and disagreements were resolved by discussion.

Statistical analysis

Appropriate RCTs were included, and Review Manager Version 5.1 (The Cochran Collaboration, Oxford, England) was used for preparation of the review. Stata 12.0 software (StataCorp, College Station, TX, United States) was used for statistical analysis. The risk ratio of data was estimated by the Mantel-Haenszel χ^2 method, where P values < 0.05 were considered significantly different. Study heterogeneity was evaluated by Cochran I^2 statistics, where I^2 < 50% indicated a lack of heterogeneity. If significant heterogeneity was found, a random effects model was applied for evaluation of the pooled data; otherwise, a fixed effects model was used. Possible publication bias was assessed by Egger's and Begg's funnel plots, where P values < 0.05 indicated little publication bias.

RESULTS

Twelve RCTs met the inclusion criteria, and characteristics of each study are presented in Table 1. In total, there were 2403 enrolled participants in the trials who were treated with 5-HT agonists, GABA-B receptor agonists, dopamine-receptor antagonists, and placebo control. Combination 5-HT agonist and PPI therapy was given in seven trials, combination GABA-B receptor agonist and PPI therapy in four trials, and combination dopamine-receptor antagonist and PPI therapy in one trial. In all RCTs, monotherapy was directly compared with combination PPI therapy. In the 5-HT agonist studies, the doses of PPI and mosapride or cisapride were the same across patients. However, in the GABA-B receptor agonist studies, different kinds of PPI and variable doses of baclofen or lesogaberan were used. All trials included mild to moderate GERD patients, with severe participants divided into a subgroup. The primary endpoints evaluated in these trials were symptom or endoscopic response, and the relief score was used to determine the symptomatic remission.

Symptom response

Table 2 details the symptom response in ten studies. Six trials[2,13,20,25-27] compared the addition of mosapride or cisapride to PPI therapy to PPI alone therapy, and four trials[23,24,28,29] compared baclofen or lesogaberan to placebo PPI control. There was no statistically significant difference in symptom response between combined therapy and single therapy in these ten trials (95%CI: 1.0-1.2, P = 0.05) (Figure 1A). Furthermore, we divided those ten trials into a 5-HT agonist group and a GABA-B recep-

tor agonist group and found that neither group displayed significant differences between combination and monotherapy for symptom response (95%CI: 1.0-1.2, $P = 0.21$; 95%CI: 0.8-1.7, $P = 0.40$) (Figure 1B and C).

Symptom score change

The 5-HT receptor agonist group showed a change in symptom score in the two treatment groups, even though the symptom assessments were different. Since Ndraha[29] and Hsu *et al*[2] used the frequency scale for the symptoms of gastroesophageal reflux (FSSG) score, we combined the two trials to assess the change in symptom score. Combination therapy yielded more symptomatic relief relative to monotherapy (95%CI: 2.1-3.0, $P < 0.00001$) (Figure 1D). Although symptom response in these two treatment groups was not statistically different, the clinical symptoms in the combination therapy group were relieved more than the single therapy group. Overall, these findings suggest that combined therapy may have improved patient quality of life.

Endoscopic response

To explore the mucosal healing in RE patients, we investigated the endoscopic response in two trials[19,25] where endoscopic response was reported. Overall, the endoscopic response in RE patients was not significantly different between 5-HT agonist and PPI combined therapy and PPI single therapy (95%CI: 0.7-2.6, $P = 0.44$) (Figure 1E).

Reflux wave amplitude and wave duration

Two trials[24,29] reported LESP, reflux wave amplitude, and wave duration. As shown in Figure 1F, combined therapy may reduce reflux wave amplitude [95%CI: -6.0-(-1.8), $P = 0.0003$] but not wave duration (95%CI: -0.4-0.6, $P = 0.65$) (Figure 1G). Taken together, these findings suggest that combined therapy in GERD may reduce the number of reflux episodes but not the duration of acid exposure time.

Proportion of adverse effects

Combined prokinetic and PPI therapy may be linked to additional side effects, such as reflux, abdominal pain, indigestion, diarrhea, chest pain, and constipation. Since only six of the 12 trials reported adverse effects, we only included these studies in our proportional analysis. The side-effects ratio demonstrated that side effects were elevated in patients with combined relative to single PPI therapy (95%CI: 1.06-1.36, $P = 0.005$) (Figure 1H). To further explore the side-effects of the 5-HT group, we excluded the GABA-B receptor agonists group. However, we found no difference between the two therapies for the 5-HT agonist group (95%CI: 0.84-1.39, $P = 0.53$) (Figure 1I). Single side-effects ratio in the GABA-B receptor agonist group was evaluated, and there were significantly more side effects in the GABA-B receptor agonists combined group than in the group with PPI therapy alone (95%CI: 1.1-1.5, $P = 0.004$) (Figure 1J).

Publication bias

As shown in Figure 2, no publication bias was detected in symptom response (Egger's test $P = 0.333$; Begg's test $P = 0.721$) or adverse event proportion (Egger's test $P = 0.246$; Begg's test $P = 0.452$).

DISCUSSION

Previous studies have reported that PPI therapy was more effective than H2R agonists and prokinetics for GERD[4], but none had investigated the efficacy of combined prokinetic and PPI therapy. In this systematic review and meta-analysis, we demonstrated that combination prokinetic and PPI therapy was no more efficacious than PPI alone for GERD. This therapy did improve patients' reported symptoms score, suggesting that it may enhance patient quality of life.

Since the 1990s, PPIs have been the mainstay treatment for GERD[31] even though a large number of patients fail to improve with a standard single PPI therapy[7]. Approximately 15% of eosinophilic esophagitis (EE) patients (mainly of Los Angeles grades C and D), 20% of Barrett's esophagus (BE) patients, 40%-50% of NERD patients[15], and up to 40% of patients with extra-esophageal manifestations of GERD[32] did not therapeutically benefit from standard PPI therapy. Recently, a number of studies found that PPIs are less effective for NERD than RE[10,13,15,33], but the underlying mechanism remains unknown.

Hiyama *et al*[34] evaluated whether *Heliobacter pylori* infection and sex may contribute to attenuated PPI efficacy in NERD. Miyamoto *et al*[33] identified younger age, constipation, and GI dysmotility as potential influencing factors of PPI non-responsiveness in NERD. Adding a prokinetic to PPI may partly alleviate symptoms of NERD, but there is little evidence available regarding an impact on mucosal healing[35]. However, Koshino *et al*[16] demonstrated that mosapride (15 mg/d) did not change salivary secretion and esophageal motility in healthy volunteers.

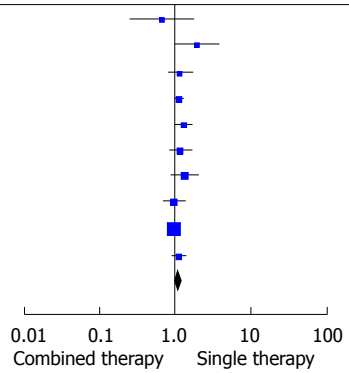
There are available different PPIs for the treatment of GERD, including omeprazole, lansoprazole, rabeprazole, pantoprazole, esomeprazole, and others. Meta-analyses failed to reveal a difference in efficacy for symptom relief among various PPIs[36]. Prokinetics in addition to PPI therapy may be a new treatment paradigm for PPI-non responsive patients. The GABA-B agonists, baclofen and lesogaberan, were reported to be effective in treating GERD by reducing LES pressure, LES relaxations, and acid reflux episodes[37]. Unfortunately, its clinical use is limited by side effects, including dizziness and constipation[9].

Here, we analyzed 12 RCTs to determine the therapeutic benefit of combination prokinetic and PPI therapy over PPI therapy alone. We found no significant difference between these two groups regarding symptom or endoscopic response. When we divided these 12 trials into two groups, according to 5-HT agonist and GABA-B agonist, we still did not find any difference between the combination and single therapy groups. Regarding adverse events, GABA-B agonists, but not 5-HT agonists, were associated with increased frequency of adverse effects. In

A

Study or subgroup	Combined therapy Events	Total	Single therapy Events	Total	Weight	Risk ratio M-H, fixed, 95%CI
Beaumont *et al*[27], 2009	4	12	6	12	1.7%	0.67 [0.25, 1.78]
Boeckxstaens *et al*[22], 2011	21	104	11	105	3.1%	1.93 [0.98, 3.79]
Cho *et al*[24], 2013	19	24	13	19	4.1%	1.16 [0.80, 1.67]
Hsu *et al*[2], 2010	39	40	41	47	10.7%	1.12 [0.99, 1.26]
Madan *et al*[19], 2004	25	28	23	33	6.0%	1.28 [0.99, 1.66]
Miwa *et al*[12], 2011	43	86	33	77	9.9%	1.17 [0.84, 1.63]
Shaheen *et al*[28], 2013	110	458	22	122	9.9%	1.33 [0.88, 2.01]
Vakil *et al*[23], 2013	110	240	21	44	10.1%	0.96 [0.68, 1.35]
van Rensburg *et al*[25], 2001	120	173	129	177	36.3%	0.95 [0.83, 1.09]
Vigneri *et al*[26], 1995	31	35	28	35	8.0%	1.11 [0.90, 1.36]
Total (95%CI)		1200		671	100.0%	1.10 [1.00, 1.20]
Total events	522		327			

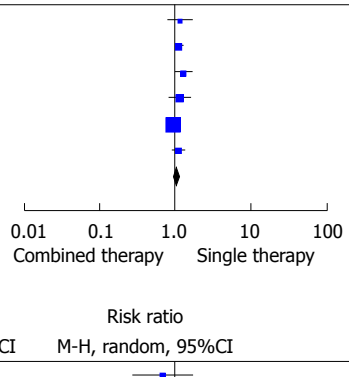
Heterogeneity: Chi^2 = 11.10, df = 9 (P = 0.27); I^2 = 19%
Test for overall effect: Z = 1.98 (P = 0.05)



B

Study or subgroup	Combined therapy Events	Total	Single therapy Events	Total	Weight	Risk ratio M-H, fixed, 95%CI
Cho *et al*[24], 2013	19	24	13	19	5.5%	1.16 [0.80, 1.67]
Hsu *et al*[2], 2010	39	40	41	47	14.3%	1.12 [0.99, 1.26]
Madan *et al*[19], 2004	25	28	23	33	8.0%	1.28 [0.99, 1.66]
Miwa *et al*[12], 2011	43	86	33	77	13.2%	1.17 [0.84, 1.63]
van Rensburg *et al*[25], 2001	120	173	129	177	48.4%	0.95 [0.83, 1.09]
Vigneri *et al*[26], 1995	31	35	28	35	10.6%	1.11 [0.90, 1.36]
Total (95%CI)		386		388	100.0%	1.06 [0.97, 1.15]
Total events	277		267			

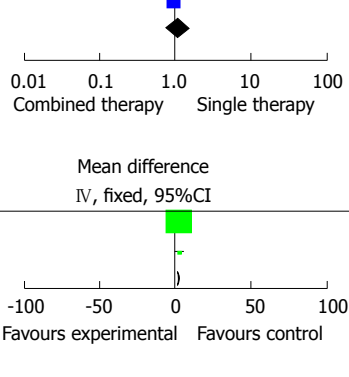
Heterogeneity: Chi^2 = 6.05, df = 5 (P = 0.30); I^2 = 17%
Test for overall effect: Z = 1.26 (P = 0.21)



C

Study or subgroup	Combined therapy Events	Total	Single therapy Events	Total	Weight	Risk ratio M-H, random, 95%CI
Beaumont *et al*[27], 2009	4	12	6	12	10.6%	0.67 [0.25, 1.78]
Boeckxstaens *et al*[22], 2011	21	104	11	105	18.0%	1.93 [0.98, 3.79]
Shaheen *et al*[28], 2013	110	458	22	122	29.8%	1.33 [0.88, 2.01]
Vakil *et al*[23], 2013	164	240	31	44	41.6%	0.97 [0.79, 1.20]
Total (95%CI)		814		283	100.0%	1.16 [0.81, 1.66]
Total events	299		70			

Heterogeneity: Tau^2 = 0.07; Chi^2 = 6.88, df = 3 (P = 0.08); I^2 = 56%
Test for overall effect: Z = 0.80 (P = 0.43)



D

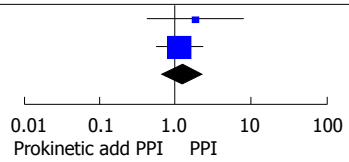
Study or subgroup	Prokinetic add on PPI mean	SD	Total	PPI alone mean	SD	Total	Weight	Mean difference IV, fixed, 95%CI
Hsu *et al*[2], 2010	13.42	1.16	44	10.85	1.03	50	96.7%	2.57 [2.12, 3.02]
Ndraha *et al*[29], 2011	7.5	5.9	30	4.6	3.3	30	3.3%	2.90 [0.48, 5.32]
Total (95%CI)			74			80	100.0%	2.58 [2.14, 3.02]

Heterogeneity: Chi^2 = 0.07, df = 1 (P = 0.79); I^2 = 0%
Test for overall effect: Z = 11.53 (P < 0.00001)

E

Study or subgroup	Prokinetic add PPI Events	Total	PPI Events	Total	Weight	Odds ratio M-H, fixed, 95%CI
Madan *et al*[19], 2004	12	17	6	11	15.0%	2.00 [0.41, 9.71]
van Rensburg *et al*[25], 2001	123	136	135	152	85.0%	1.19 [0.56, 2.55]
Total (95%CI)		153		163	100.0%	1.31 [0.66, 2.61]
Total events	135		141			

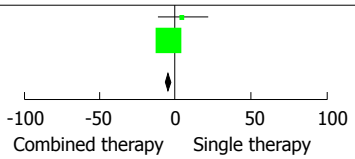
Heterogeneity: Chi^2 = 0.33, df = 1 (P = 0.56); I^2 = 0%
Test for overall effect: Z = 0.78 (P < 0.44)



F

Study or subgroup	Combined therapy mean	SD	Total	Single therapy mean	SD	Total	Weight	Mean difference IV, fixed, 95%CI
Cho *et al*[24], 2013	89.1	29.1	24	83.1	31	19	1.3%	6.00 [-12.16, 24.16]
Smythe *et al*[30], 2003	44	2	12	48	3	11	98.7%	-4.00 [-6.10, -1.90]
Total (95%CI)			36			30	100.0%	-3.87 [-5.96, -1.78]

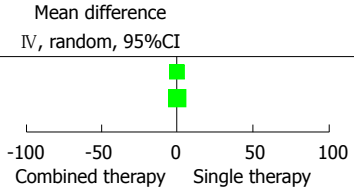
Heterogeneity: Chi^2 = 1.15, df = 1 (P = 0.28); I^2 = 13%
Test for overall effect: Z = 3.63 (P = 0.0003)



G

Study or subgroup	Combined therapy mean	SD	Total	Single therapy mean	SD	Total	Weight	Mean difference IV, random, 95%CI
Cho *et al*[24], 2013	3.8	0.7	24	3.4	0.6	19	42.5%	0.40 [0.01, 0.79]
Smythe *et al*[30], 2003	2.9	0.1	12	3	0.1	11	57.5%	-0.10 [-0.18, -0.02]
Total (95%CI)			36			30	100.0%	0.11 [-0.37, 0.60]

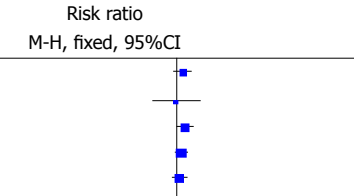
Heterogeneity: Tau^2: = 0.10; Chi^2 = 6.08, df = 1 (P = 0.01); I^2 = 84%
Test for overall effect: Z = 0.45 (P = 0.65)



H

Study or subgroup	Combined therapy Events	Total	Single therapy Events	Total	Weight	Risk ratio M-H, fixed, 95%CI
Boeckxstaens *et al*[22], 2011	55	122	45	122	16.9%	1.22 [0.90, 1.66]
Miwa *et al*[12], 2011	11	98	11	97	4.2%	0.99 [0.45, 2.17]
Shaheen *et al*[28], 2013	216	521	43	140	25.5%	1.35 [1.03, 1.77]
Vakil *et al*[23], 2013	228	368	46	87	28.0%	1.17 [0.95, 1.45]
van Rensburg *et al*[25], 2001	72	173	66	177	24.6%	1.12 [0.86, 1.45]
Vigneri *et al*[26], 1995	1	35	2	35	0.8%	0.50 [0.05, 5.27]
Total (95%CI)		1317		658	100.0%	1.20 [1.06, 1.36]
Total events	583		213			

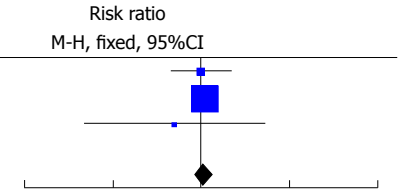
Heterogeneity: Chi^2 = 1.86, df = 5 (P = 0.87); I^2 = 0%
Test for overall effect: Z = 2.79 (P = 0.005)



I

Study or subgroup	Combined therapy Events	Total	Single therapy Events	Total	Weight	Risk ratio M-H, fixed, 95%CI
Miwa *et al*[12], 2011	11	98	11	97	14.1%	0.99 [0.45, 2.17]
van Rensburg *et al*[25], 2001	72	173	66	177	83.3%	1.12 [0.86, 1.45]
Vigneri *et al*[26], 1995	1	35	2	35	2.6%	0.50 [0.05, 5.27]
Total (95%CI)		306		309	100.0%	1.08 [0.84, 1.39]
Total events	84		79			

Heterogeneity: Chi^2 = 0.52, df = 2 (P = 0.77); I^2 = 0%
Test for overall effect: Z = 0.63 (P = 0.53)



J

Study or subgroup	Combined therapy Events	Total	Single therapy Events	Total	Weight	Risk ratio M-H, fixed, 95%CI
Boeckxstaens *et al*[22], 2011	55	122	45	122	24.0%	1.22 [0.90, 1.66]
Shaheen *et al*[28], 2013	216	521	43	140	36.2%	1.35 [1.03, 1.77]
Vakil *et al*[23], 2013	228	368	46	87	39.7%	1.17 [0.95, 1.45]
Total (95%CI)		1011		349	100.0%	1.25 [1.08, 1.45]
Total events	499		134			

Heterogeneity: Chi^2 = 0.68, df = 2 (P = 0.71); I^2 = 0%
Test for overall effect: Z = 2.91 (P = 0.004)

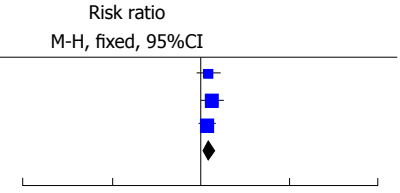


Figure 1 Meta-analysis. A: Symptom response in 5-hydroxytryptamine (5-HT) and GABA-B receptor therapies; B: Symptom response in the 5-HT receptor agonist group; C: Symptom response in the GABA-B receptor agonist group; D: Symptom score change (FSSG) in the two therapies; E: Endoscopic response in 5-HT and GABA-B receptor therapies; F: Wave amplitude in 5-HT and GABA-B receptor therapies; G: Wave duration in 5-HT and GABA-B receptor therapies; H: Adverse events proportion in 5-HT and GABA-B therapies; I: Adverse events in 5-HT agonist group; J: Adverse events in GABA-B receptor agonist group.

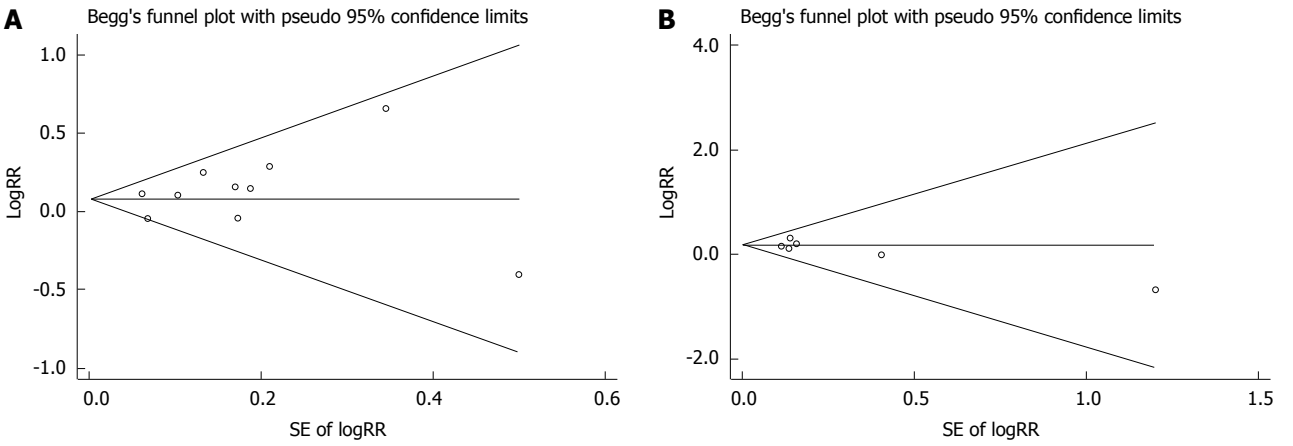


Figure 2 Funnel plots for publication bias in meta-analysis. A: No publication bias was detected in symptom response (Egger's test P = 0.333; Begg's test P = 0.721); B: Adverse event proportion (Egger's test P = 0.246; Begg's test P = 0.452).

terms of symptom score change, combined therapy may improve patient quality of life by decreasing the number of reflux episodes, although acid exposure time was unaltered.

There are some limitations of our meta-analysis to consider. First, PPI and prokinetic therapies in the 12 trials were not identical. Although one study found little impact on symptom response[36], we cannot rule out the possibility of treatment course affecting this measure. To limit this complication, we only chose studies for this analysis with a treatment course for GERD longer than two weeks. Second, an inherent weakness of all systematic reviews and meta-analyses is the possibility that some studies failed to find significant symptom improvement in the peer-reviewed literature[38], thereby leading us to underestimate the main effect. To overcome these limitations, long-term RCTs need to be performed with a larger quantity of participants to more effectively determine efficacy and safety profiles of combined prokinetic and PPI therapy.

In summary, patients with GERD respond to combined prokinetic and PPI therapy. Combination therapy may improve patient quality of life, although there was no significant difference in symptom or endoscopic responses. Side effects of combined therapy may be greater than single therapy, especially with GABA-B agonists. Whether prokinetic plus PPI is indeed therapeutically efficacious for GERD will require future trials.

COMMENTS

Background

Gastroesophageal reflux disease (GERD) is a common disease, affecting individuals of all nationalities. The standard treatment regimen is proton pump inhibitors (PPIs). Despite this therapy, many patients remain symptomatic. The addition of prokinetics to PPI therapy may improve the symptoms of GERD in these patients, but the efficacy and safety of prokinetics remain to be established.

Research frontiers

This meta-analysis was performed to assess the efficacy and safety of PPI mono-therapy versus combined therapy in patients with GERD. The main measured outcomes are as follows: symptom response, symptoms score change, endoscopic response, wave amplitude, wave duration, and adverse events.

Innovations and breakthroughs

Authors found with this meta-analysis no demonstrable effect of either combination therapy for relief of symptoms or alteration in endoscopic response. However, with combination therapy there was a greater symptom score change, suggesting that this therapy did improve patient quality of life.

Applications

Authors' results suggest that combination therapy may have some advantages for symptomatic or endoscopic response relative to PPI alone. There is some evidence that combination therapy may partially improve patient quality of life. Until further randomized controlled trails with a large population number are carried out, authors suggest use of combination therapy on an individual basis.

Terminology

FSSG score: a questionnaire given to GERD patients in order to assess severity of symptoms, based on a frequency scale for symptoms of GERD.

Peer review

The efficacy and safety for the use of prokinetics plus PPI compared to PPI monotherapy for GERD remain unclear. Therefore, the authors conducted a meta-analysis to determine the efficacy and safety of these two treatment regimens for GERD. The authors concluded that combination therapy may partially improve the patient quality of life (symptoms score change, reflux wave amplitude, and wave duration).

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CASE REPORT

Peliosis hepatis complicated by portal hypertension following renal transplantation

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Abstract

Peliosis hepatis (PH) is a vascular lesion of the liver that mimics a hepatic tumor. PH is often associated with underlying conditions, such as chronic infection and tumor malignancies, or with the use of anabolic steroids, immunosuppressive drugs, and oral contraceptives. Most patients with PH are asymptomatic, but some present with abdominal distension and pain. In some cases, PH may induce intraperitoneal hemorrhage and portal hypertension. This study analyzed a 46-year-old male who received a transplanted kidney nine years prior and had undergone long-term immunosuppressive therapy following the renal transplantation. The patient experienced progressive abdominal distention and pain in the six months prior to this study. Initially, imaging studies revealed multiple liver tumor-like abnormalities, which were determined to be PH by pathological analysis. Because the hepatic lesions were progressively enlarged, the patient suffered from complications related to portal hypertension, such as intense ascites and esophageal varices bleeding. Although the patient was scheduled to undergo liver transplantation, he suffered hepatic failure and died prior to availability of a donor organ.

Key words: Peliosis hepatis; Liver neoplasm; Portal hypertension; Renal failure; Renal transplantation

Core tip: Peliosis hepatis (PH) is a vascular lesion of the liver that mimics a hepatic tumor. PH has been associated with the use of anabolic steroids or immunosuppressive drugs. Although most patients remain asymptomatic, some patients suffer from PH-related portal hypertension complications. This case study describes a 46-year-old man who underwent long-term immunosuppressive therapy following renal transplantation. Upon development of abdominal distention, PH was diagnosed by pathological analysis. Ultimately, the patient suffered hepatic failure when the immunosuppressive agent was withdrawn and died.

Yu CY, Chang LC, Chen LW, Lee TS, Chien RN, Hsieh MF, Chiang KC. Peliosis hepatis complicated by portal hypertension following renal transplantation. *World J Gastroenterol* 2014; 20(9): 2420-2425 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i9/2420.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i9.2420

INTRODUCTION

Peliosis hepatis (PH) is a rare vascular lesion of the liver, characterized by cystic blood-filled cavities distributed throughout the parenchyma of liver. The term originates

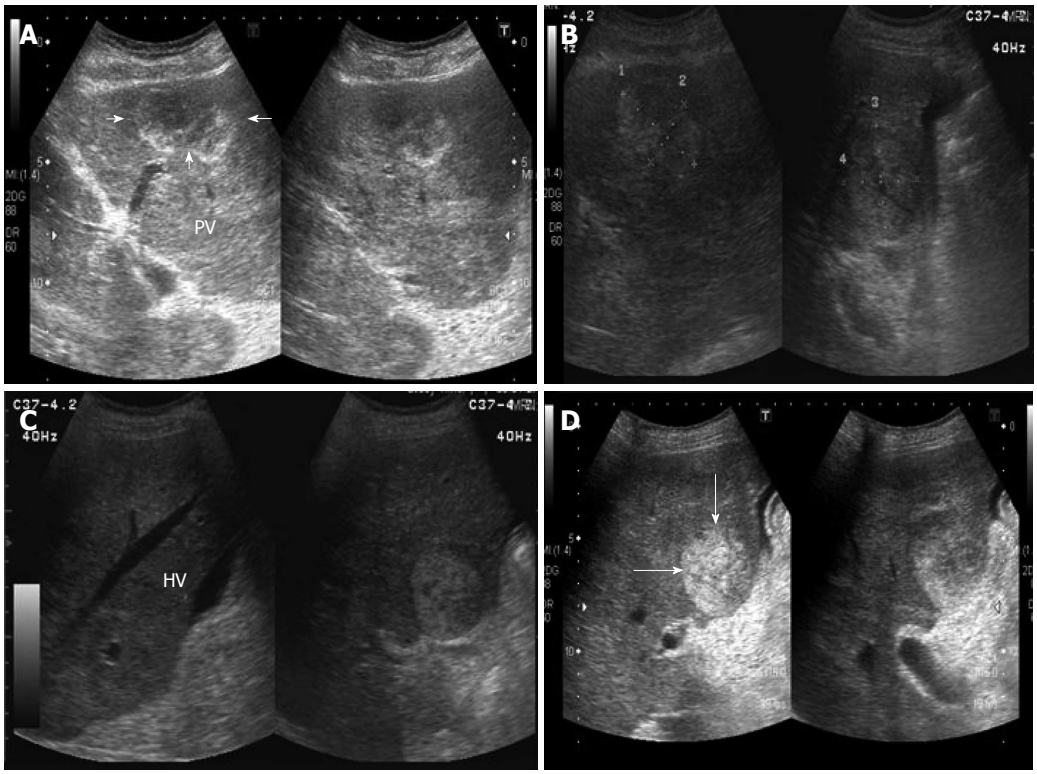

Figure 1 Abdominal ultrasound showing multiple hyperechoic or mixed echoic tumors in the bilateral lobes of liver (arrows). Minimal ascites was also detected. A: One mixed echoic tumor (arrows) at segment 3, left portal vein (PV); B: One mixed echoic tumor (marked) at segment 5; C: One hyperechoic tumor at segment 5, right hepatic vein (HV), minimal ascites also detected; D: One hyperechoic tumor (arrows) at segment 5 near the main portal trunk.

from the Greek word "pelios", which means blue/black or discolored extravasated blood. Peliosis is most commonly found in the liver, but can also involve the spleen, pancreas, lungs, and other organs[1,2]. The epidemiology of PH is poorly characterized, in part because PH is often only identified as an incidental finding on abdominal imaging or *via* autopsy[3]. The pathogenesis of PH includes sinusoidal cell proliferation, which obstructs blood flow and causes pre-sinusoidal portal hypertension[4]. PH has also been described in patients with hematologic malignancies, infection with pulmonary tuberculosis, and human immunodeficiency virus infection, as well as those taking anabolic steroids, immunosuppressive drugs, and oral contraceptives[3,5-13]. Although most patients remain asymptomatic or have slowly progressive disease, some patients develop PH-related portal hypertension complications, such as esophageal variceal bleeding or intense ascites[14,15]. Here, we describe the case of a 46-year-old male renal transplant recipient with PH-related complications.

CASE REPORT

A 46-year-old male received a transplanted kidney for end-stage renal disease nine years prior to this study, after which he received long-term immunosuppressive therapy [mycophenolate mofetil (180 mg/tab twice a day), tacrolimus (1 mg/tab three times a day), and prednisolone (5 mg/tab daily)] to prevent renal graft rejection. The patient had a history of alcohol consumption (consumed five or more beverages per day) and smoking for thirty years, but had no family history of renal disease, hypertension, diabetes mellitus, or liver malignancy. The patient presented to the emergency department with complaint of pain in the upper right quadrant of his abdomen, which he described as dull, radiating to the back, and persisting for several hours. The pain was unrelated to food intake or a change in posture, and there were no aggravating or relieving factors. The associated symptoms were nausea and vomiting. The patient did not report any fever, night sweating, jaundice, diarrhea, tarry stool, or body weight loss.

While in the emergency room, the following vital signs were recorded: the patient's blood pressure was 140/80 mmHg, pulse rate was 113 beats per minute, respiratory rate was 20 breaths per minute, and body temperature 37.3 ℃. A physical examination revealed no abnormalities outside of the abdominal area, where distended abdomen and hypoactive bowel sounds were observed. The right side of the abdomen was tender and the dull pain shifted upon abdominal percussion. The liver span was 16 cm in the right midclavicular line, and the spleen was not palpable. In addition, a superficial engorged vein was found on the abdominal wall. The patient's laboratory data showed abnormal liver biochemistry tests, including: aspartate aminotransferase: 62 U/L, alanine aminotransferase: 108 U/L, total bilirubin: 1.4 mg/dL, and creatinine: 3.89 mg/dL. The patient had normocytic anemia (hemoglobin: 11.2 g/dL), normal platelet count (238000/mm^3), and normal prothrombin time.

An abdominal ultrasound revealed multiple hyperechoic or mixed echoic tumors in the bilateral lobes of

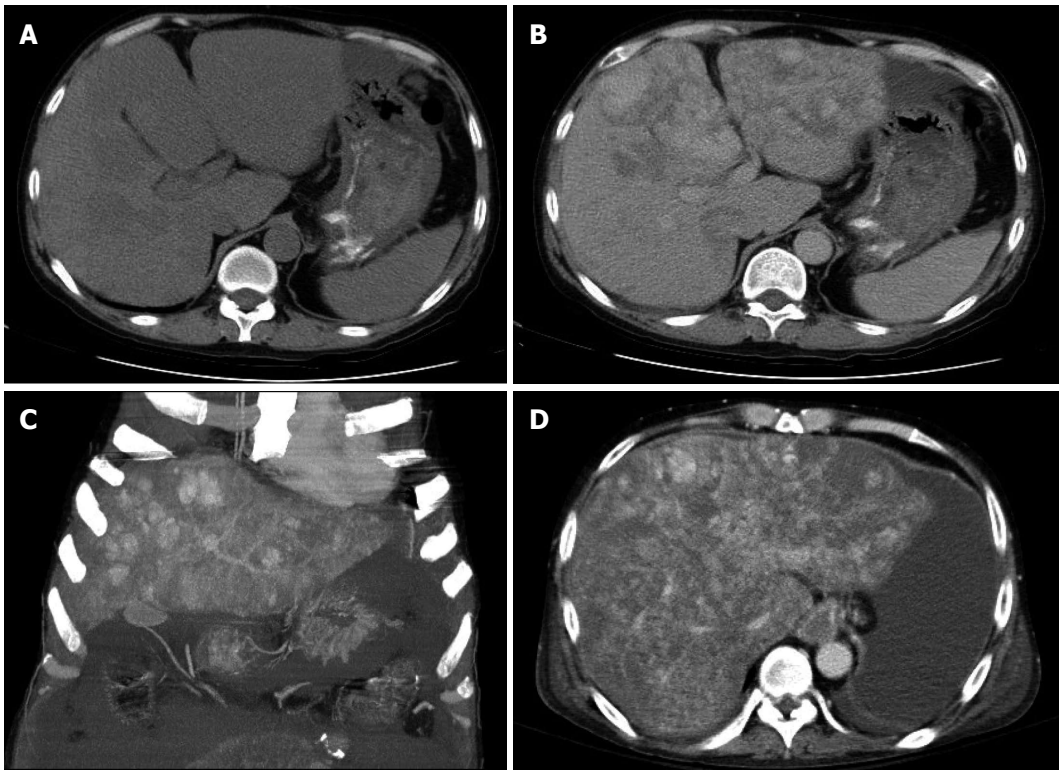

Figure 2 Liver dynamic computed tomography showing multiple liver tumors. A: Hypodense liver tumors before contrast injection; B: Heterogeneous enhancement in the arterial phase and portal venous phase after contrast injection; C: Follow-up computed tomography showing increased size of the hepatic lesion; D: Massive ascites observed at 4 mo after the follow-up.

the liver (Figure 1); minimal ascites fluid was detected. Liver dynamic computed tomography (CT) also indicated the presence of multiple liver tumors. The liver tumors were hypodense before contrast injection (Figure 2A) and showed heterogeneous enhancement in the arterial phase and portal venous phase after contrast injection (Figure 2B). The liver tumor showed no sign of contrast washout in the delayed phase of CT. The patient continued to suffer from persistent abdominal pain and abdominal distension during hospitalization. An ultrasound-guided fine needle biopsy was performed on the tumors in liver segment four, where no ascites accumulated between the peritoneum and liver border. However, only fragmented hepatic tissue specimens and a large amount of blood fluid were obtained from the procedure.

The ascites fluid that was aspirated from the lower abdomen was yellowish and clear. The serum ascites albumin gradient exceeded 1.1 mg/dL, indicating portal hypertensive type ascites. Histological analysis of the liver biopsy and cell block cytology specimens revealed no malignancies. Microscopic analysis of the liver biopsy specimens revealed sinusoidal dilatation with red blood cells and endothelial cells within the sinusoidal cavity (Figure 3A, B). Additional immunohistochemical analysis of biopsy specimens revealed that the sinusoidal endothelial cells were diffusely positive for CD34 and CD31 (Figure 3C, D). The biopsy specimens showed positive staining for Ki-67, a RNA transcription factor and nuclear protein selectively expressed in proliferating cells, which is used to measure cell proliferation within the tissue. The cell proliferative Ki-67 stain index was within 20% to 40%, suggesting active cell proliferation (Figure 3E).

The differential diagnoses from these pathologic findings included infection, hemangioma, and PH. Bacterial and viral infections were excluded based on the negative results of bacterial culture and serum antibody studies for the Epstein-Barr virus, cytomegalovirus, and human immunodeficiency virus. The rapidly increasing size of the tumor was not consistent with hemangioma, thus this diagnosis was also excluded. Due to the exclusion of these alternatives and the clinical presentation of the lesion, PH was the most likely diagnosis. The patient suffered from portal hypertension-related complications, including massive ascites (Figure 2C, D) and recurrent bleeding esophageal varices. Although the patient was scheduled to undergo a liver transplantation, the variceal bleeding, intense ascites, and hepatic encephalopathy led to death prior to availability of a donor organ.

DISCUSSION

In the renal transplant setting, PH can occur after transplantation, rather than being a manifestation of chronic kidney disease[11]. Possible causes of PH development post-transplantation include the use of immunosuppressive drugs, such as azathioprine and cyclosporine, or the development of an opportunistic infection[11,13,16]. For example, PH incidence can be reduced by administration of

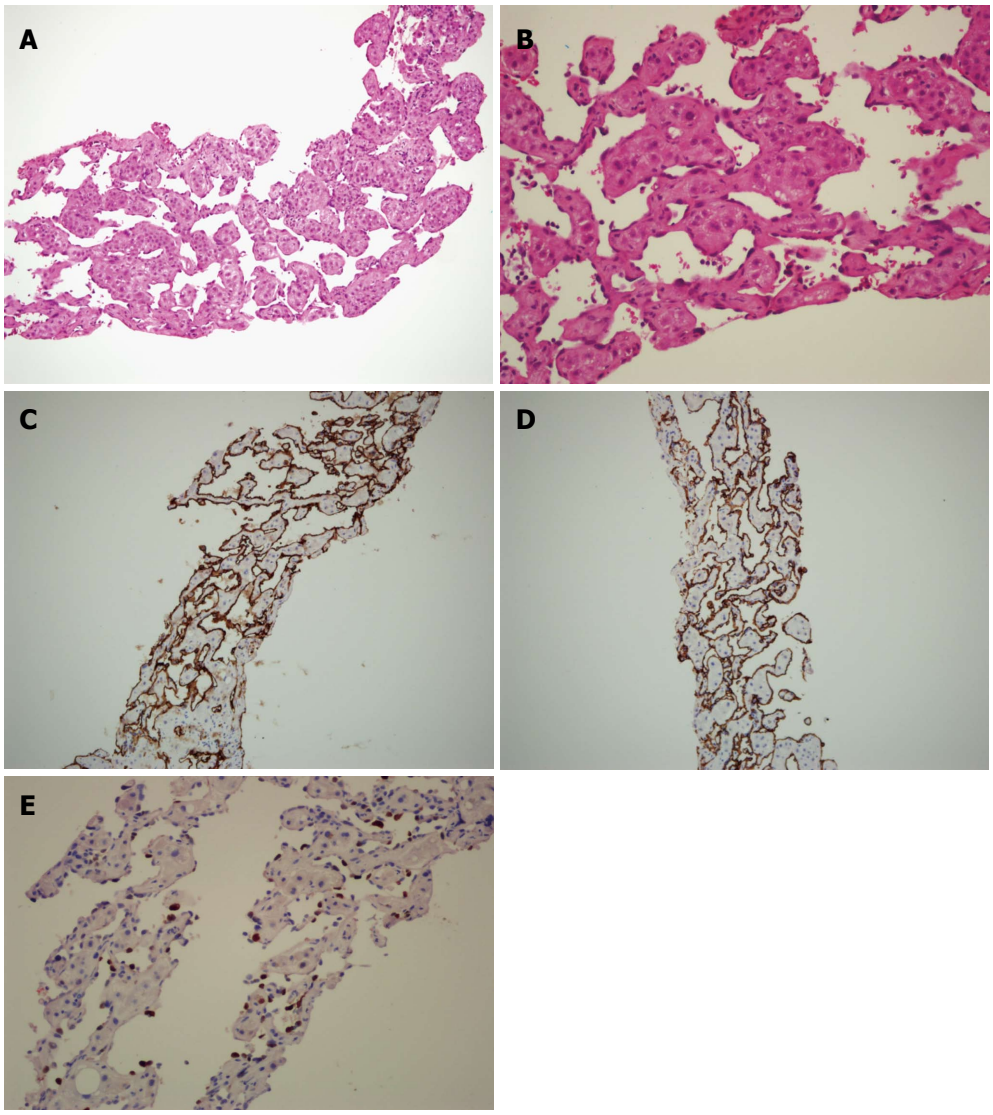

Figure 3 Microscopic analysis of liver biopsy specimens showing sinusoidal dilatation with red blood cell-filled cysts without endothelial lining cells. A: 100 × magnification of hematoxylin-eosin stained biopsy specimens; B: 400 × magnification of A; C, D: Immunohistochemistry analysis of CD31 (C) and CD34 (D) in liver biopsy specimens showing diffuse positive staining in the sinusoid endothelial cells; E: Ki-67 staining of liver biopsy specimens. The cell proliferative Ki-67 stain index was within 20%-40%, indicating active cell proliferation in the tissue.

sulphonamide therapy to prevent *Pneumocystis carinii* infection[17]. Some drugs known to cause PH, including 6-thioguanine, oxaliplatin and urethane, can induce sinusoidal endothelial cell damage, consistent with the pathology of the patient described herein. However, other drugs associated with PH, such as anabolic steroids, glucocorticoids, methotrexate, vitamin A and oral contraceptives, do not affect sinusoidal endothelial cells.

The risk factors for the patient in this study may have been related to his post-renal transplantation status and long-term use of immunosuppressive agents. However, there are no current reports associating mycophenolate mofetil or tacrolimus with sinusoidal endothelial cell damage. Another PH risk factor for this patient was alcohol consumption, which can contribute to glutathione depletion. Sinusoidal endothelial damage has been associated with glutathione depletion[13], and glutathione levels play an essential role for immunosuppressant detoxification in sinusoidal endothelial cells. Thus, given the patient's alcohol consumption and the use of several immunosuppressive drugs, glutathione depletion was a major risk factor for hepatic sinusoidal endothelial damage.

Lesions of PH are often asymptomatic or associated with abnormal liver biochemical test levels, but more severe complications exist, such as progressive fibrosis, cirrhosis, and portal hypertension[3,7-9,16,18,19]. Hepatic lesions may regress upon withdrawal of immunosuppressive drugs, but this approach substantially increases the risk of transplant rejection[3]. As a result, this strategy has not been adequately evaluated. In this specific patient, immunosuppressive therapy could not be discontinued because of the risk of renal graft failure, thus increasing the risk of hepatic lesions.

By dynamic CT imaging, PH typically presents with hypoattenuating lesions before contrast injection, but with variable enhancement patterns after contrast injec-

tion. In the arterial phase, the lesions may present with progressive enhancement in the centrifugal or centripetal direction. In the delayed phase, the lesion may show a diffuse, increased attenuation. The differential diagnoses for these lesions include abscesses, hemangiomas, and hypervascular metastases[20]. Pyogenic hepatic abscesses appear as a low-density lesion with peripheral enhancing after contrast injection. Some septa, pyogenic fluid, or gas within the lesion are also seen. It is important to distinguish a hepatic abscess from PH and to avoid inadvertent aspiration of the periotic lesions, which may be fatal[19].

Two histologic types of PH have been reported, a parenchymal type and a phlebectatic type. The parenchymal type is characterized by hemorrhagic parenchymal necrosis and congestion of the lining of cavities with hepatocytes. The phlebectatic type is characterized by endothelial cells lining the cavity and aneurysmal dilation of the central vein[4]. In our patient, the pathological analysis of the liver biopsy specimen revealed dilated sinusoidal space with red blood cells and endothelial lining cells in the sinusoidal cavity, consistent with the phlebectatic form of PH. Increased cell proliferation, as indicated by Ki-67 staining, was detected in our patient. Because there are no previous reports that have analyzed the Ki-67 index in a case of PH, the diagnosis of well-differentiated angiosarcoma cannot be completely excluded for our case given the status of cellular hyperproliferation.

The treatment of PH varies between individuals. Serial follow-up imaging studies may be adequate for asymptomatic patients with slow progressive disease. Furthermore, removing exacerbating factors, such as certain drugs or infection, may halt the progression of PH. For patients with localized lesions, a hepatectomy can be performed to remove the lesions[6,9,10,12,21]. For patients with rapidly progressing PH and additional complications, liver transplantation may be the optimal therapeutic option[22].

In conclusion, PH is a rare vascular lesion of the liver in patients receiving kidney transplantation and is mainly associated with prolonged use of certain immunosuppressants. Patients with PH are often asymptomatic, but may develop progressive fibrosis, cirrhosis, and portal hypertension. Hepatic failure can develop if immunosuppressive therapy cannot be withdrawn, which likely contributed to the outcome of the specific case described herein.

COMMENTS

Case characteristics

The abdominal distension and pain were the main symptoms in the case.

Clinical diagnosis

Diagnosis is suspected with imaging techniques and assessed by liver biopsy.

Differential diagnosis

The differential diagnoses for these lesions include abscesses, hemangiomas, hypervascular metastases and angiosarcoma and all of them can be distinguished by clinical conditions and CT images but except angiosarcoma.

Laboratory diagnosis

Abnormal liver biochemical test levels can be the initial presentation but the thrombocytopenia, hypoalbuminemia and hyperbilirubinemia can be founded when portal hypertension and hepatic failure develop.

Imaging diagnosis

By dynamic computed tomography imaging, peliosis hepatis (PH) typically presents with hypoattenuating lesions before contrast injection, but with variable enhancement patterns after contrast injection. In the arterial phase, the lesions may present with progressive enhancement in the centrifugal or centripetal direction. In the delayed phase, the lesion may show a diffuse, increased attenuation.

Pathological diagnosis

Microscopic analysis of the liver biopsy specimens revealed sinusoidal dilatation with red blood cells and endothelial cells within the sinusoidal cavity.

Treatment

Removing exacerbating factors, such as certain drugs or infection, may halt the progression of PH and for patients with localized lesions, a hepatectomy can be performed to remove the lesions.

Term explanation

The term “peliosis” originates from the Greek word "pelios", which means blue/black or discolored extravasated blood.

Experiences and lessons

The peliosis hepatis was founded in patient with specialized risk factors and it may go to hepatic failure even to death.

Peer review

Hepatic peliosis is a rare condition mimicking hepatic tumors. The natural history of this disease is variable with little knowledge in the literature. Therefore, the case is interesting. The references have been updated.

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CASE REPORT

Intestinal obstruction due to migration of a thermometer from bladder to abdominal cavity: A case report

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Abstract

Intraperitoneal foreign bodies such as retained surgical instruments can cause intestinal obstruction. However, intestinal obstruction due to transmural migration of foreign bodies has rarely been reported. Here, we report a case of intestinal obstruction due to a clinical thermometer which migrated from the bladder into the abdominal cavity. A 45-year-old man was admitted to our hospital with a one-year history of recurrent lower abdominal cramps. Two days before admission, the abdominal cramps aggravated. Intestinal obstruction was confirmed with upright abdominal radiography and computerized tomography scan which showed dilation of the small intestines and a thermometer in the abdominal cavity. Then laparotomy was performed. A scar was observed at the fundus of the bladder and a thermometer was adhering to the small bowels and mesentery which resulted in intestinal obstruction. Abdominal cramps were eliminated and defecation and flatus recovered soon after removal of the thermometer.

Key words: Intestinal obstruction; Foreign body; Thermometer; Transmural migration; Bladder

Core tip: Intestinal obstruction due to transmural migration of foreign body is a rare condition. Here we report a case of intestinal obstruction due to transmural migration of a thermometer from the bladder to the abdominal cavity. The patient recovered soon after removal of the thermometer.

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INTRODUCTION

Intraperitoneal foreign bodies are common events in children, alcoholics, psychiatric patients and criminals[1]. They can be categorized as either intraluminal or extraluminal of gastrointestinal tract. Intraluminal ones are usually ingested coins, buttons, pins, batteries and so on[2]. They can be lodged in narrow areas (*i.e.,* pylorus and ileocecal valve), resulting in obstruction or perforation[1]. Extraluminal ones, such as retained surgical sponges, are infrequently encountered in patients who have been treated with prior surgery or some other interventional medical procedures. They can lead to intestinal obstruction or abscess formation with or without secondary bacterial

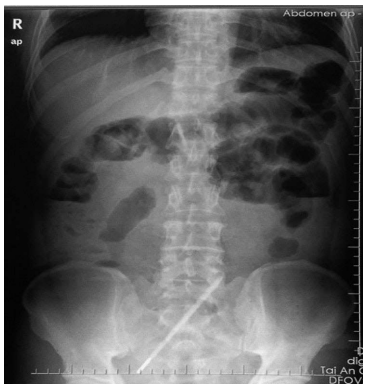

Figure 1 Abdominal X-ray showed dilation of the small intestines, gas-fluid levels and a thermometer-like object in the abdominal cavity.

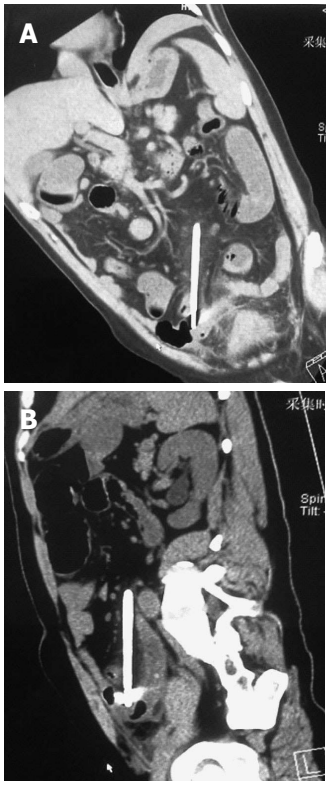

Figure 2 Computerized tomography scan revealed thin walls of intestines and a thermometer-like object in the lower quadrant of the abdomen (A, B).

infection[3]. Foreign bodies can be detected by abdominal radiography and computerized tomography (CT)[4]. Here we report a rare case of intestinal obstruction due to transmural migration of a thermometer from the bladder to the abdominal cavity.

CASE REPORT

In April 2013, a 45-year-old man was admitted to our hospital with a one-year history of recurrent lower abdominal cramps. The symptoms could be alleviated with antibiotic treatment. Two days before admission, the abdominal cramps aggravated, and defecation and flatus stopped. On physical examination, he had a regular pulse

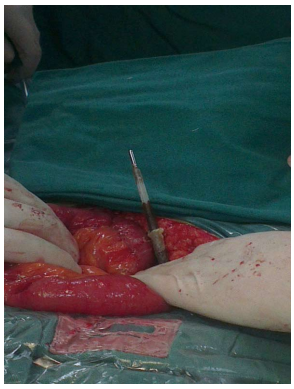

Figure 3 Thermometer coated with pus was seen pressing on the small bowels and mesentery.

of 82 beats/min, a respiratory rate of 20 breaths/min, and a temperature of 37.6 ℃. His cardiopulmonary and neurologic examinations were normal. Abdominal examination revealed distension with lower abdomen tenderness and rebound tenderness. Abdominal rumbling sound was weak. Blood routine test revealed a white blood cell count of 8.78 × 10^9/L, neutrophil 87.41%, lymphocyte 8.3%, hemoglobin concentration 12.8 g/dL, and platelet count 173 × 10^9/L. The serum level of C-reactive protein was 75.1 mg/L. Abdominal radiography showed dilation of the small intestines, gas-fluid levels and a thermometer-like object in the abdominal cavity (Figure 1). CT scan revealed thin walls of the intestines and a thermometer-like object in the lower quadrant of abdominal cavity (Figure 2). He was diagnosed with intestinal obstruction.

The patient was addicted to alcohol. When he was drunk, he would behave strangely. He admitted that he had inserted a thermometer into the urinary passage through the penis two years ago without referring to a doctor. Subsequently, he experienced increased urinary frequency and urgency and enlarged testicles with pain. For this, he would take antibiotics to control the pain.

After the examinations, the patient was sent to the operation room immediately and exploratory laparotomy was performed *via* lower abdominal midline incision. After opening the abdomen, much purulent exudative fluid was sucked out. A scar was observed at the fundus of the bladder and a thermometer was adhering to the small bowels and mesentery, resulting in intestinal obstruction (Figure 3). Three hundred milliliters of normal saline was injected to the bladder through a urinary catheter during the operation and no fluid exuded from around the scar. Abdominal cramps were eliminated and defecation and flatus recovered soon after removal of the thermometer.

DISCUSSION

This is a case report of intraperitoneal foreign body that led to intestinal obstruction. This patient denied ingestion of foreign bodies or prior surgical surgery. We found that the obstruction was due to a thermometer migrating from the bladder to the abdominal cavity. When there is a breakage on the bladder wall, bacteria can travel between

the urinary passage and the abdominal cavity, leading to urinary infection and intra-abdominal inflammation, which can be alleviated with antibiotic therapy as seen in our patient. The scar at the fundus of the bladder is the evidence of perforation. Furthermore, our patient was alcoholic with psychiatric problems. He admitted inserting a thermometer into urinary passage two years before.

Urinary system foreign bodies, especially bladder ones, are frequently encountered. Most of them come from the urinary tract that are inserted by the patients themselves because of being curious about sex and genital organ[5-7]. But perforation of bladder is rarely reported. It manifests as increased urinary frequency and urgency, hematuria, abdominal pain, *etc*. In our case, fortunately, the thermometer was not broken, otherwise it would cause severe clinical events, such as intestinal perforation and mercury poisoning[8,9].

To our knowledge, this is the first case report of intestinal obstruction due to transmural migration of a thermometer from the bladder to the abdominal cavity. Based on our experience, a thorough history of patients should be acquired to confirm the ingestion or insertion of any foreign bodies. If patients showed signs of intestinal obstruction, surgery should be performed to avoid further complications.

COMMENTS

Case characteristics

A 45-year-old man presented with recurrent lower abdominal cramps.

Clinical diagnosis

Abdominal distension with lower abdomen tenderness and rebound tenderness.

Differential diagnosis

Intestinal neoplasm, abdominal abscess, and urinary tract infection.

Laboratory diagnosis

WBC 8.78 × 10^9/L, Neutrophil 87.41%, and CRP 75.1 mg/L.

Imaging diagnosis

Abdominal radiography and computerized tomography scan showed dilation of the small intestines and a thermometer-like object in the abdominal cavity.

Treatment

Exploratory laparotomy was performed and a thermometer was removed.

Related reports

This is the first case report of intestinal obstruction due to transmural migration of a thermometer from bladder to abdominal cavity.

Experiences and lessons

Foreign object detained in the bladder can penetrate into the abdominal cavity and cause intestinal obstruction and peritonitis.

Peer review

This case report describes a rare condition that intestinal obstruction occurred due to a clinical thermometer which migrated from the bladder into the abdominal cavity.

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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