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Contents

Weekly Volume 19 Number 46 December 14, 2013

Contents

World Journal of Gastroenterology
Volume 19 Number 46 December 14, 2013

World Journal of Gastroenterology
Volume 19 Number 46 December 14, 2013

Contents

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EDITORIAL

Liver physiology and liver diseases in the elderly

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Abstract

The liver experiences various changes with aging that could affect clinical characteristics and outcomes in patients with liver diseases. Both liver volume and blood flow decrease significantly with age. These changes and decreased cytochrome P450 activity can affect drug metabolism, increasing susceptibility to drug-induced liver injury. Immune responses against pathogens or neoplastic cells are lower in the elderly, although these individuals may be predisposed to autoimmunity through impairment of dendritic cell maturation and reduction of regulatory T cells. These changes in immune functions could alter the pathogenesis of viral hepatitis and autoimmune liver diseases, as well as the development of hepatocellular carcinoma. Moreover, elderly patients have significantly decreased reserve functions of various organs, reducing their tolerability to treatments for liver diseases. Collectively, aged patients show various changes of the liver and other organs that could affect the clinical characteristics and management of liver diseases in these patients.

Key words: Liver disease; Aging; Physiology; Immunology

Core tip: The morphology and physiology of the liver changes with aging and an understanding of those changes is important for the management of liver diseases. We first summarized the various changes in the liver with aging. We then reviewed the reported characteristics of liver diseases found in the elderly. This kind of information could be increasingly important in the near future, because the proportion of the world's population over 60 years old is increasing, especially in developed countries.

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INTRODUCTION

The proportion of the world's population over 60 years old is increasing, especially in developed countries. Morphology and functions of the liver as well as other organs change with aging. Understanding these changes is important for the management of liver diseases in the elderly. In addition, the pathogenesis of many liver diseases is immune-mediated, and immune systems also change with aging, affecting the clinical picture of liver diseases.

CHANGES IN LIVER MORPHOLOGY AND FUNCTIONS WITH AGING

Morphology of the aged liver and microscopic or molecular characteristics of senescent hepatocytes

In general, liver volume is reduced by 20%-40% in the elderly, with these reductions more marked in women (up to 44% decline) than in men[1]. Microscopically, elderly subjects have elevated numbers of hepatocytes with increased ploidy. Hepatocytes show decreased numbers of

mitochondria but increased volume of individual mitochondria, although functional impairment of mitochondria has not been demonstrated. Hepatocytes in elderly subjects contain denser body compartments, such as secondary lysosomes and lipofuscin, than do hepatocytes in younger subjects[2]. Lipofuscin accumulation has been associated with chronic oxidative stress and a failure to degrade damaged and denatured proteins[3]. Moreover, accumulating evidence suggests that lipofuscin interferes with cellular pathways due to its ability to trap metallic cations and facilitate further free radical formation[4].

Vacuolation of hepatocyte nuclei has been associated with diabetes mellitus and non-alcoholic fatty liver disease. However, vacuolated hepatocyte nuclei were recently shown to be more abundant in senescent hepatocytes expressing p21 or γH(2)AX[5], suggesting they are a marker of hepatocyte senescence. Moreover, increased size of hepatocyte nuclei in nonalcoholic fatty liver disease (NAFLD) has been associated with telomere shortening and p21 upregulation[6], suggesting that increased nuclear size is also a marker of hepatocyte senescence.

Cellular senescence is associated with aberrant activation of oncogenes, and senescent pre-malignant hepatocytes have been found to secrete cytokines and chemokines through interactions with their environment, resulting in immune-mediated clearance of these cells. Impairment of immune surveillance has been associated with the development of hepatocellular carcinoma (HCC)[7]. This scenario could account for the preferential development of HCC in aged patients with chronic liver diseases, irrespective of the etiology of these diseases[8].

Recently, resistin, an adipokine that inhibits phosphorylation of AMP-activated protein kinase and modulates insulin resistance, has been shown to induce senescence-associated β-galactosidase in mouse hepatocytes[9]. Resistin has been shown to act by inhibiting the function of sirtuin 1, one of the 7 members of the sirtuin family of histone deacetylases shown to act as crucial negative regulators during the aging process[9].

Molecular changes during hepatocyte senescence should be clarified in more detail in the near future. The identification of senescence-causing factors may be beneficial in preventing senescence-associated liver diseases.

Blood flow

Liver blood flow is estimated to be decreased by 35%-50% in the elderly, and may be responsible for age-related reductions in liver volume[10].

Hepatic function

Liver function tests: Although interindividual differences have been observed, liver functions are relatively well preserved in elderly individuals. Hepatic enzymes and high-density lipoprotein cholesterol are well maintained, while bilirubin levels may decline with age due to reductions in muscle mass and hemoglobin concentrations[11]. Moreover, age was reported to be associated with modest decreases in albumin and γ-glutamyl transpeptidase concentrations, and increases in bilirubin concentration, after adjustments for sex, alcohol use, and components of the metabolic syndrome, suggesting that liver function may be decreased in these individuals[12].

Alanine aminotransferase (ALT) concentrations have been reported to decrease with age in both men and women, independent of components of the metabolic syndrome. These findings suggest the need to identify an optimal cut-off point for normal ALT in elderly patients[12].

Drug metabolism

Phase I hepatic metabolism (first-pass hepatic uptake) of drugs has been reported to be decreased in the elderly, possibly due to reduced liver volume and hepatic blood flow, leading to a decline in hepatic drug metabolism. Metabolism of drugs with low phase I hepatic metabolism is likely to be impaired mainly by liver volume reduction.

A previous report suggested that drug metabolism is reduced by up to 30% after 70 years of age, and that a reduction in liver cytochrome P450 may also contribute to decreased drug metabolism. Cytochrome P450 activity was shown to be 32% lower in subjects > 70 years than in subjects aged 20-29 years[13].

Liver regeneration

Liver regeneration capacity has been reported to decline with age[14,15]. The mechanisms underlying the reductions in regeneration capability are complex. One of these mechanisms involves a decrease in the concentrations of circulating epidermal growth factor (EGF), with the response of hepatocytes to EGF also reduced due to age-associated loss of EGF receptors or deficits in signaling after EGF binds to its receptor[16]. Another mechanism underlying reduced hepatocyte proliferation capacity may be the inhibition of cyclin-dependent kinases by interaction with the chromatin remodeling protein Bim, which is expressed in aged hepatocytes[17]. Along with reductions in regenerative capacity, telomere length has been reported reduced in aged livers, especially in patients with liver diseases[18].

Immune system

Many liver diseases are mediated by the host immune response. Therefore, changes in immune functions may affect the clinical picture of various liver diseases. Several changes in the immune system have been observed in elderly individuals.

Innate immunity: Most of the immune cells involved in innate immunity, such as monocytes/macrophages and natural killer (NK) cells, show decreased function with aging[19]. Although the percentage and number of $CD56^{bright}$ NK cells gradually decline with age, the percentage and number of $CD56^{dim}$ NK cells progressively increase[20].

In addition, dendritic cells (DCs), which are the most potent antigen-presenting cells, show significant function-

al changes with aging. DCs play pivotal roles in the onset and regulation of adaptive immune responses and control the state of tolerance to self-antigens[21]. Immature DCs promote tolerance through induction of regulatory T cells (Treg), whereas mature DCs stimulate effector T cells. DCs in the elderly show inappropriate maturation induced by infections or tissue injury, which may lead to alterations in the balance between the tolerogenic and immunogenic functions of DCs and instigate the development of autoimmune diseases[22].

Adoptive immunity: T cell number and diversity of repertoire are decreased and T cell expansion, differentiation, and signaling intensity are impaired with aging. The numbers of $CD4^+$ T cells are decreased, while the numbers of $CD8^+$ T cells are increased. The expression of the costimulatory molecule CD28 is decreased on T cells, impairing their ability to proliferate and secrete interleukin-2[23]. Treg function is decreased after age 50 years, which may be associated with the increases in autoimmunity[24].

The numbers of B cell precursors in the bone marrow (pre-B cells), as well as peripheral B cells, decrease with age[25]. In contrast, immunoglobulin concentrations may increase with age[26], but the quantities of specific antibodies and the diversity of the B cell repertoire decrease[27].

In summary, immune responses against foreign antigens and malignant cells seem to be impaired with age because of the reductions in number and functions of most immunocompetent cells. In contrast, the decrease in Tregs and the impairment of DC maturation may result in a predisposition to autoimmunity.

LIVER DISEASES IN THE ELDERLY

The prevalence of some liver diseases increases with aging, and advanced liver disease is observed more often in older than in younger patients. Moreover, various physiological changes associated with aging may affect the pathogenesis of liver diseases. In addition, the decreased reserve capacity of most organs in elderly individuals may impair their ability to manage liver diseases.

Viral hepatitis

Hepatitis A: Acute hepatitis A virus (HAV) infection is usually self-limiting. However, elderly patients with acute HAV infection experience hepatocellular dysfunction with frequent jaundice and coagulopathy, as well as an increased incidence of complications, such as prolonged cholestasis, pancreatitis, and ascites[28]. Higher hospitalization and mortality rates have been reported in elderly patients with HAV. For example, during an outbreak of HAV infection in the United States, 42% of patients aged 70 years or older required hospitalization compared with 3%-20% of adults aged 40-49 years[29]. Age-related differences in outcomes were also reported, with death rates of 0.004% in individuals aged 5-14 years and 2.7% in those older than 49 years[30]. More recent data from the Centers for Disease Control and Prevention (CDC, 2009 Surveillance) also indicate that mortality due to HAV increases with age, with no fatalities reported in patients younger than 34 years of age. The mortality rates have been estimated to be 0.05 per 100000 patients aged 45-54 years, and 0.11 per 100000 patients older than 75 years. Vaccination for hepatitis A should, therefore, be considered for people, especially the elderly, who plan to travel to endemic areas[30].

Hepatitis B: Acute hepatitis B virus (HBV) infection is uncommon in the elderly because the opportunities for HBV infection are estimated to be low in this population. However, hepatitis B and hepatitis C infections have been reported in aged residents of nursing homes[31,32]. Risk factors include sharing bath brushes, non-disposable syringes, and shaving blades, as well as sexual contact. Clinical manifestations of acute HBV infection are similar to those in younger adults. During an outbreak of acute HBV in elderly nursing home residents, most infected patients were asymptomatic, and no patient died or required hospitalization during the outbreak[31]. However, the rate of progression to chronic hepatitis B is higher in elderly than in younger patients. A report on an outbreak of acute HBV infection in a nursing home showed that 59% of patients older than 65 years of age developed chronic infection[31]. This may be due to a decreased immune response to the pathogens.

In chronic HBV infection, the prevalence rates of HBeAg and HBsAg are inversely related to patient age during the natural course of HBV infection. The prevalence rates of HBsAg in Taiwanese men and of HBeAg among HBsAg-positive men older than 60 years of age were reported to be 12.5% and 5.5%, respectively, while prevalence rates in patients aged 30-39 years were 23.8% and 23.3%, respectively[33]. Serum HBV DNA levels were found to vary by country and to be associated with HBeAg or HBV genotype[34]. Older age and male sex, in addition to serum HBV DNA levels, are regarded as risk factors not only for progression to cirrhosis[35], but the development of HCC[36].

Nucleos(t)ide analogs are effective in treating HBV infected patients, with similar efficacy in the elderly as in younger patients[37]. Although interferon-based therapy may also be effective for the treatment of chronic HBV infection, its therapeutic effects are inferior in elderly patients[38].

Hepatitis C: The prevalence of hepatitis C virus (HCV) infection varies by age because HCV infection is transmitted by blood contact, such as blood transfusion (especially before 1992), military service, intravenous drug use, tattoos, hemodialysis, and health care work. In the United States, the prevalence of HCV infection is highest in patients aged 40-49 years (4.3%), whereas those aged 60-69 years and 70 years or older have lower prevalence rates of 0.9% and 1%, respectively[39,40]. A European

study showed that the prevalence of genotype 1 HCV increases with age, being 57% in patients aged < 65 years, 72% in those aged 65-80 years, and 84% in patients older than 80 years of age[41]. Older age at the time of infection, but not duration of infection, has been associated with fibrotic progression[41] and hepatocarcinogenesis[42]. Normal ALT levels are more likely observed in elderly than in younger HCV-infected patients (46% *vs* 10.6%, respectively)[43]. However, older patients often show more fibrosis regardless of serum ALT levels[41]. Moreover, the incidence of hepatocellular carcinoma increases with aging both in hepatitis C[42] and hepatitis B[44] infected patients. Therefore, progression of fibrosis and development of hepatocellular carcinoma should be considered, especially in elderly patients with chronic viral hepatitis.

Powerful regimens have been established for the treatment of chronic HCV infection, including pegylated interferon and ribavirin, have been established. However, adverse effects are observed more often in older patients[45]. The sustained viral response rate has been shown lower in elderly than in younger patients (46% *vs* 69.7%, respectively), perhaps due to the high proportion of elderly patients who stop antiviral therapy due to side effects[46].

Hepatitis E: The prevalence of hepatitis E virus (HEV) infection differs markedly in endemic and non-endemic areas. However, recent reports suggested that exposure to HEV occurs frequently in Western countries. In the United States, 16% of blood donors younger than 60 years of age were positive for anti-HEV IgG, compared with 25.5% of those older than 60 years[47]. Furthermore, 3% of patients with acute liver injury suspected of being drug-induced liver injury were seropositive for anti-HEV IgM. Most patients with serology consistent with acute HEV infection were older than 60 years of age[48].

Autoimmune liver disease

The prevalence rates of autoimmune liver diseases, including autoimmune hepatitis (AIH) and primary biliary cirrhosis (PBC), are relatively high in older patients, whereas primary sclerosing cholangitis is more common in those in the third or fourth decade of life[49-51]. However, the results of laboratory tests associated with these autoimmune liver diseases are not associated with age, and treatment strategies are usually identical in older and younger patients.

AIH: Almost 20% of patients develop AIH after 60 years of age, and the disease is frequently progressive and unexpected because ascites and cirrhosis are common manifestations at presentation with few other symptoms[49,52,53]. Most elderly patients respond well to corticosteroid therapy[52]. Rates of treatment failure are lower in older than in younger patients (5% *vs* 24%), and elderly patients have lower rates of fatality from liver failure or need for liver transplantation (5% *vs* 21%)[52,53]. Notably, elderly patients are at risk of treatment-related complications, especially osteopenia and compression fracture[54]. Furthermore, they may have other comorbidities and medication requirements that complicate their management.

PBC: Advancing age has been associated with poor prognosis in patients with PBC, and elderly patients diagnosed with PBC at a young age are likely to show a poor prognosis[55]. In contrast, patients with PBC diagnosed after 65 years of age are less likely to have progressive or advanced disease[56]. Two types of phenotypic expression of PBC were recently reported: the classical asymptomatic onset in middle-late age with mild biochemical activity, and symptomatic onset at a younger age with high biochemical activity[57]. Administration of ursodeoxycholic acid, which is the only recommended therapy for PBC, appears to be safe and has few side effects. Osteoporosis should also be considered, especially in elderly patients.

Alcoholic liver disease: Alcohol consumption is common in the elderly. In a study of individuals in the United Kingdom, 62% of subjects aged 60-92 years were drinkers, with 13% of men and 2% of women being heavy drinkers[58]. Elderly people presenting with alcoholic liver disease (ALD) had more advanced disease than younger patients[59]. Half of the elderly patients who develop cirrhosis die within 1 year of diagnosis[60]. In patients with HCV infection, alcohol drinking was associated with accelerated disease progression[61]. Adverse effects of benzodiazepines as treatment for withdrawal symptoms, such as drowsiness, fatigue, confusion, ataxia, falls and incontinence, are more common with increasing age[62].

NAFLD: NAFLD is a disease predominantly seen in middle-aged to older people. A significant proportion of cryptogenic cirrhosis may be due to the end-stage of NAFLD, and age has been reported to be a risk factor for liver fibrosis and higher mortality rate in patients with NAFLD[63]. Older patients have significantly more risk factors for NAFLD, including hypertension, obesity, diabetes, and hyperlipidemia[64]. A study of 351 consecutive patients in the United Kingdom found that albumin, alanine aminotransferase (ALT), ALT/aspartate aminotransferase ratio, and platelet counts were significantly reduced with advancing age[64]. Thus, aged patients with NAFLD are considered to have advanced liver disease.

Recently, sirtuin 1, a negative regulator of aging, was reported to play key roles in the regulation of lipid and glucose homeostasis[65]. This finding suggested that aging was associated with the development of NAFLD, and that activating sirtuin 1 may be a novel therapeutic strategy for patients with NAFLD. Several molecular characteristics of hepatocyte senescence have been observed in patients with NAFLD, with hepatocyte senescence being closely associated with advanced fibrosis stage and poor clinical outcome[66]. Thus, the development and patho-

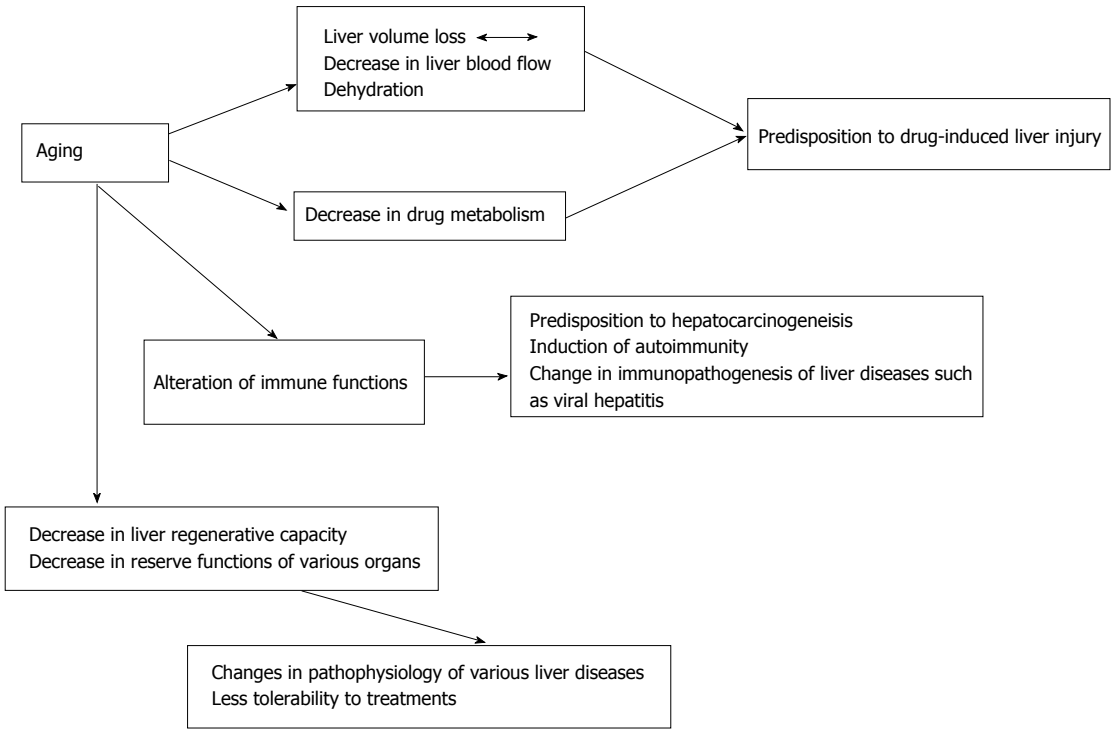

Figure 1 Physiological changes in elderly subjects associated with the development or pathophysiological modification of liver diseases. Aging is associated with decreases in liver volume, blood flow, drug metabolism and regenerative capacity, and alterations in immune functions. Changes due to decreased reserve functions of various organs could affect the clinical characteristics and management of liver diseases in the elderly.

genesis of NAFLD may be closely associated with the aging process.

Drug-induced liver injury: Old age is a risk factor of drug-induced liver injury (DILI) because the elderly are more susceptible to adverse drug reactions[67]. Moreover, patients over 75 years old required significantly longer hospitalization for DILI[68]. In contrast, a recent report suggested that older age is associated with a cholestatic type of liver injury[69], and a study in Japan also showed rates of cholestatic liver injury was higher in patients > 65 than < 65 years of age (46% *vs* 31.6%).

Elderly patients may receive many types of drugs for treatment of comorbid conditions. For example, a Japanese study of patients with DILI showed that elderly patients > 75 years of age were taking significantly more concomitant drugs at the time of liver injury[68]. Other reports from Western countries also suggested greater drug usage among elderly patients. For example, a study of 466 patients in Germany > 70 years of age found that these patients were receiving an average of 3.7 prescribed medicines in addition to 1.4 over-the-counter medications daily[70]. In a prospective study in the Netherlands, 94.2% of elderly patients, mean age 82.3 years, were taking more than one drug, and 73.3% were prescribed four or more drugs[71]. Several pharmacokinetic and pharmacodynamic mechanisms that may predispose a patient on multiple medications to an increased risk of DILI have been proposed[72]. Adverse effects of both the individual drugs and their synergistic interactions must be taken into consideration in elderly patients.

Liver tumor: HCC is more common in elderly patients with liver cirrhosis[73]. Elderly patients were reported to develop HCC even without fibrosis[74], suggesting that aging itself may be a predisposing factor for hepatocarcinogenesis. The impact of viral eradication on HCC prevention was found to be less significant in older than in younger patients chronically infected with HCV, especially in patients at an advanced stage of liver disease[75]. These observations indicate the need for long-term follow-up of elderly patients with chronic HCV infection, even after viral eradication and especially in male patients with liver cirrhosis.

Hepatic resection for HCC can be performed safely and effectively in elderly patients[76]. Regional therapies, such as radiofrequency ablation and transarterial chemoembolization, may also be considered for elderly patients with HCC, if liver function and tumor stage are acceptable[77].

Liver transplantation: The proportion of adult liver transplantation recipients in the United States older than 60 years of age increased from 10% in 1990 to more than 20% by 1999[78]. Some problems remain to be addressed regarding liver transplantation in elderly patients. Increased age has been associated with a poorer survival rate[79,80], although other studies suggested that advanced age alone should not be a contraindication for liver transplantation[81,82]. Among 2141 patients who underwent

Table 1 Clinical characteristics of liver diseases in patients

Liver diseases	Characteristics
Viral hepatitis	
Hepatitis A	Higher hospitalization and mortality rates
Hepatitis B	More likely to progress to chronic hepatitis or cirrhosis
Hepatitis C	More likely to progress fibrosis
	Higher rates of hepatocellular carcinoma development
	Decreased tolerability to treatment
Autoimmune diseases	
Autoimmune hepatitis	Sometimes progressive
Primary biliary cirrhosis	Higher rates of treatment-related complications
	Sometimes progressive
	More likely to have osteoporosis
Alcoholic liver disease	Progressive
Nonalcoholic fatty liver disease	Higher prevalence
	Progressive
Hepatocellular carcinoma	Higher rates of development

retransplantation, more than 10% were over 60 years of age[82]. Being over 60 years of age was not independently associated with an increase in mortality when adjusted for factors that were found to influence survival[82]. Elderly patients may have multiple risk factors, including coronary artery disease or malignancy, and face age-associated quality of life impairments, such as instability, incontinence, immobility, dementia, and polypharmacy[83]. Moreover, aged recipients have a significantly lower quality of life, as assessed by physical functioning, bodily pain, general health, vitality, social functioning, role emotional, and physical component score[84]. Therefore, careful consideration is required in choosing liver transplantation for elderly patients.

CONCLUSION

Aged patients show various changes in the liver, which could affect the clinical characteristics of liver diseases in these patients (Table 1). Decreases in functioning of the liver and other organs as well as alterations in immune functions should be taken into consideration in the management of the liver diseases (Figure 1).

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TOPIC HIGHLIGHT

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Early stage colon cancer

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Abstract

Evidence has now accumulated that colonoscopy and removal of polyps, especially during screening and surveillance programs, is effective in overall risk reduction for colon cancer. After resection of malignant pedunculated colon polyps or early stage colon cancers, long-term repeated surveillance programs can also lead to detection and removal of asymptomatic high risk advanced adenomas and new early stage metachronous cancers. Early stage colon cancer can be defined as disease that appears to have been completely resected with no subsequent evidence of involvement of adjacent organs, lymph nodes or distant sites. This differs from the clinical setting of an apparent "curative" resection later pathologically upstaged following detection of malignant cells extending into adjacent organs, peritoneum, lymph nodes or other distant sites, including liver. This highly selected early stage colon cancer group remains at high risk for subsequent colon polyps and metachronous colon cancer. Precise staging is important, not only for assessing the need for adjuvant chemotherapy, but also for patient selection for continued surveillance. With advanced stages of colon cancer and a more guarded outlook, repeated surveillance should be limited. In future, novel imaging technologies (*e.g.*, confocal endomicroscopy), coupled with increased pathological recognition of high risk markers for lymph node involvement (*e.g.*, "tumor budding") should lead to improved staging and clinical care.

Key words: Colon cancer; Node-negative colon cancer; Staging of colon cancer; Nodal micrometastases; Follow-up and surveillance of early colon cancer

Core tip: Evidence has now accumulated that colonoscopy and removal of polyps, especially during screening and surveillance programs, is effective in overall risk reduction for colon cancer. After resection of malignant pedunculated colon polyps or early stage colon cancers, long-term repeated surveillance programs can also lead to detection and removal of asymptomatic high risk advanced adenomas and new early stage metachronous cancers. In future, novel imaging technologies (*e.g.*, confocal endomicroscopy), coupled with increased pathological recognition of high risk markers for lymph node involvement (*e.g.*, "tumor budding") should lead to improved staging and clinical care.

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INTRODUCTION

Adenocarcinoma of the colon, including rectum, is a major cause of morbidity and mortality among all internal malignant diseases in men and women. When the disease is at an advanced stage with documented metastatic involvement of lymph nodes or other organs, the prognosis is especially dismal. A number of different staging criteria have been used to estimate the depth of cancer penetration in the colon as well as the extent of extracolonic disease involvement. Currently, a commonly used

Table 1 Colon cancer staging

AJCC stage	TNM stage	TNM criteria
Stage 0	Tis N0 M0	Tumor confined to mucosa
Stage Ⅰ	T1 N0 M0	Tumor invades submucosa
Stage Ⅰ	T2 N0 M0	Tumor invades muscularis propria
Stage ⅡA	T3 N0 M0	Tumor invades subserosa
Stage ⅡB	T4 N0 M0	Tumor invades adjacent organs
Stage ⅢA	T1-2 N1 M0	Tumor metastases to 1-3 nodes
Stage ⅢB	T3-4 N1 M0	Tumor metastases to 1-3 nodes
Stage ⅢC	Any T, N2, M0	Tumor metastases to 4 or more nodes
Stage Ⅳ	Any T or N, M1	Metastases to distant sites

AJCC: American Joint Committee on Cancer; TNM: Tumor/Nodes/Metastases. Other classification methods include: Dukes System: A, tumor confined to intestinal wall; B, tumor invading through the intestinal wall; C, tumor with lymph node involvement; D, tumor with distant metastases; and Astler-Coller System: A, Tumor limited to mucosa; B1, Tumor through muscularis mucosa but not muscularis propria; B2, Tumor beyond muscularis propria; C1, B1 with lymph node metastases; C2, B2 with lymph node metastases; D, Distant metastases. Other criteria include: venous and lymphatic invasion and differentiation.

staging method for colon cancer is based on the TNM (tumor/node/metastases) system as delineated by the American Joint Committee on Cancer (AJCC), now with a staging manual and atlas in its 7th edition[1]. These different AJCC stages are summarized in Table 1.

EARLY STAGE COLON CANCER

Early stage colon cancer can be defined as disease that appears to have been completely resected with no subsequent evidence of involvement of adjacent organs, lymph nodes or distant sites. This definition differs from the clinical setting of an apparent "curative" resection later pathologically upstaged following detection of malignant cells extending into adjacent organs, peritoneum, lymph nodes, or other distant sites, including the liver.

This highly-selected group with disease localized in the colon still remains at especially high risk for subsequent development of colon polyps and metachronous colon cancer. Conceptually, this definition of early stage disease reflects increasing use of colonoscopic surveillance as an important tool in an emerging management approach. Precise staging, however, is critical, not only in assessing the need for adjuvant chemotherapy, but also for the selection of patients for continued surveillance. In patients with advanced stages of colon cancer and a more guarded outlook, repeated surveillance should be limited.

IMAGING METHODS

Although imaging methods are important in defining suspected areas of involvement, complete staging currently requires pathological assessment of resected tissue, particularly to define early stage disease. Usually staging has been estimated after surgical removal of the colon cancer, however, experience has shown that complete staging is also possible after endoscopic resection of a malignant pedunculated polyp that has minimal invasion. For these malignant polyps, however, deep histopathological assessment is not possible and lymph nodes are not removed. Further upstaging of colon cancer may result from employment of ultrasound, computed tomography (CT), magnetic resonance imaging or position emission tomography with pathological confirmation. In contrast, studies have already confirmed that methods such as fecal immunochemical testing (FIT) or CT have limited value in the detection of early stage colon cancer. For example, a high rate of false-negative results with FIT for early stage cancers was recently recorded[2] and CT was shown to have a low sensitivity for diagnosis of early T1 or T2 cancers[3].

Studies to explore staging using evolving endoscopic methods have also appeared. For example, a recent report[4] compared new techniques for assessment of the actual depth of colon cancer invasion. Magnification chromoendoscopy and endoscopic ultrasound were found to have similar accuracy in estimating the depth of invasion, but neither procedure was believed to currently have sufficient diagnostic accuracy for use as a reliable or recommended standard[4]. Further investigative efforts are needed to explore novel and emerging imaging developments, particularly endoscope-based or probe-based confocal endomicroscopic methods. These offer the possibility for more rapid (and possibly for economical) differentiation of neoplastic from non-neoplastic colonic disease, earlier diagnosis of colorectal cancer, further evaluation of degree of differentiation and estimation of invasion depth for early colorectal cancer[5-8].

OUTCOME OF STAGING

Evidence has accumulated to show that a more advanced cancer stage is correlated with a worse clinical outcome. In patients with localized and limited disease confined to the submucosa or muscularis propria, the overall 5 year survival is about 70%. With more advanced disease extending beyond the subserosa into adjacent structures, peritoneum, lymph nodes or distant sites, the overall 5 year survival is about 30%. Even in early stage colorectal cancer, bowel perforation from the tumor itself or anastomotic leakage following surgery is associated with increased recurrence rates and an impaired disease-free survival[9].

Early detection of colon cancer has been an important goal for physicians evaluating patients at increased risk for colon cancer. Colonoscopic regimens of surveillance have emerged based on good evidence that morbidity and mortality can be improved[10,11]. A number of guidelines have been developed for endoscopic surveillance of high risk groups to detect colon cancer. Some high risk categories have included a documented personal and family history of colon adenomas and colon cancer as well as inflammatory bowel disease. Among these high risk groups, a prior history of a completely resected colon cancer is a special group that should be considered for regular surveillance, particularly for those with early

stage disease[12]. Most important, recent publications have provided good evidence that colonoscopy is associated with reduced colorectal cancer mortality[13,14]. In addition, persistent and sustained reduction in colorectal cancer mortality has been attributed, in large part, to the effect of polypectomy[14]. For malignant colorectal polyps with localized submucosal invasion, similar long-term results have been recorded, although a risk for new colon polyps, including advanced adenomas, and metachronous colon cancer persists[15].

SURVEILLANCE AFTER COLON CANCER RESECTION

Earlier randomized clinical trials compared intense with less intense surveillance after a "curative" resection[16-20]. Unfortunately, a number of methodological flaws in these studies were noted[21], particularly the inclusion of both early- (*i.e.*, node-negative) and late- (*i.e.*, node-positive) stage disease together in the comparison groups, regardless of the intensity of later surveillance. Perhaps, in these earlier studies, evaluation of more homogeneous populations, particularly with early-stage colon cancer, would have shown a positive effect of surveillance because prognosis for patients with nodal involvement, invasion of other structures and distant metastases would be expected to be much more limited[21]. Moreover, a more recent Cochrane evaluation has suggested a survival benefit for selected patients with more intense follow-up[22]. Finally, long-term studies of symptomatic early stage colon cancer patients followed over more than 10 years[23] demonstrated no locally recurrent disease. However, in the same study[23], there was still an ongoing risk for new and asymptomatic neoplasms, including advanced adenomas and early-stage metachronous colon cancers.

RISK OF LYMPH NODE METASTASES

A number of factors critical to accurate clinical and pathological staging have been explored in recent years, especially definition of high risk factors for lymph node involvement, if only early stage colon cancer with submucosal invasion (or T1) disease appears to be present. These factors include lymphatic invasion, venous invasion, tumor budding, poor tumor differentiation, extent (especially width) of submucosal invasion, complete disruption of muscularis mucosa. Indeed, some studies have suggested that up to 16% with localized submucosal invasive disease may already have lymph node metastases[24-30].

For malignant pedunculated colon polyps, Haggitt *et al*[24] initially proposed a 4-level classification defined by increasing depths of cancer invasion into the submucosa, particularly if deeper than the polyp stalk. Level 4 invasion into the submucosa was thought to represent the highest risk for lymph node metastases. Some have used alternative measures of depth of invasion to ensure complete electrocautery removal of malignant pedunculated polyps. For example, a distance from the leading invasive margin of the cancer to the cautery line of more than 2 mm has been empirically used as a guideline of an adequate resection of a pedunculated lesion with a stalk. If the cautery line is involved with malignant cells after removal of a malignant polyp, colectomy should be done.

For non-polypoid malignant lesions with submucosal invasion, assessment is more difficult. In these, level 4 invasion was traditionally defined[24]. Others have suggested a different classification schema, especially for surgically-resected specimens, defined by submucosal depth of invasion (*i.e.*, specifically, sm1, sm2, sm3) with greatest depth of invasion having greatest risk for lymph node involvement[27,31]. For endoscopic resection, complete removal of the submucosa may be more difficult pathologically to define, although a retrospective evaluation of colorectal cancer initially treated with endoscopic resection suggested that a positive vertical (rather than lateral) resection margin and inadequate lifting sign were positively correlated with risk of residual tumor and lymph node metastases[32]. Other pathological risk factors for node metastases have also been emphasized include venous or lymphatic invasion, moderately or poorly differentiated tumor grade, tumor "budding" at the submucosal invasive front of the cancer, or a completely cancer-disrupted muscularis mucosa[33]. A high CEA value may also be predictive of metastatic disease[34,35]. Because of this increased risk for node involvement after endoscopic resection with these high risk factors, colectomy may be recommended to ensure complete cancer removal and permit more detailed node sampling for metastatic disease.

TUMOR BUDDING AND OTHER RISK FACTORS

"Tumor budding" is an independent prognostic indicator of risk for lymph node involvement, especially in early TNM stage colorectal cancer, as recently emphasized by expert pathologists[36]. This description of "tumor budding" was attributed to Imai who first postulated that this particular pathological feature of an invasive colon cancer represented a sudden or rapid growth of the leading or invasive edge of a carcinoma, in part, related to an interaction between epithelial and mesenchymal elements at the tumor margin[36]. Evidence has accumulated that tumor budding as well as high tumor grade or lymphovascular invasion are independent risk factors for lymph node metastases in patients with submucosally invasive colon cancer[37,38]. Patients with none of these high risk pathological features had only rare lymph node metastases (less than 1%) whereas the risk increased substantially with one (*i.e.*, about 20%) or multiple (*i.e.*, almost 40%) risk factors. In addition, this study showed that absence of extensive, particularly lateral, submucosal invasion (specifically, < 4 mm in width and < 2 mm in depth), had no apparent risk of metastases to lymph nodes (using an-

ti-cytokeratin immunohistochemical staining method for detection of lymph node micrometastases) if other high risk markers were absent. Similar observations have been independently reported[39-42], including a recent evaluation following endoscopic removal of submucosal invasive T1 colorectal cancers[43].

In future, the clinical relevance of other clinical and pathological methods of evaluation for staging, including stage II colon cancer, will need additional evaluation. These include number of lymph nodes surgically harvested[44-47], techniques used for lymph node evaluation (including detection of micrometastases with novel immunohistochemical stains and polymerase chain reaction methods)[48-51] as well as definition of the precise role of sentinel node mapping for node sampling[52-54] and final staging.

CONCLUSION

Colonoscopy screening and surveillance have a documented benefit in reducing the risk of colon cancer. As a result, more early stage colon cancers will be detected in surveillance programs and treated with endoscopic methods. Emerging imaging technologies, such as confocal endomicroscopic methods, may lead to further refinements in definition of patients with early stage disease as well their management. Pathological staging to define early stage disease also continues to evolve, particularly with the increased recognition of risk factors for lymph node disease in early stage colon cancers and immunohistochemical methods for lymph node evaluation, especially detection of lymph node micrometastases.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Evidence-based appraisal of the upfront treatment for unresectable metastatic colorectal cancer patients

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Abstract

Colorectal cancer (CRC) is a significant health problem, with around 1 million new cases and 500000 deaths every year worldwide. Over the last two decades, the use of novel therapies and more complex treatment strategies have contributed to progressively increase the median survival of patients with unresectable advanced CRC up to approximately 30 mo. The availability of additional therapeutic options, however, has created new challenges and generated more complicated treatment algorithms. Moreover, several clinically important points are still in debate in first-line, such as the optimal treatment intensity, the most appropriate maintenance strategy, the preferred biologic to be used upfront in patients with KRAS wild-type CRC, and the need for more detailed information on tumor biology. In this moving landscape, this review analyses why the first-line treatment decision is crucial and how the choice may impact on further treatment lines. In addition, it focuses on results of major phase Ⅲ randomized trials.

Key words: Colorectal cancer; Chemotherapy; Angiogenic inhibitors; Epidermal growth factor receptor inhibitors; Maintenance; First-line

Core tip: The choice of the first-line therapy is crucial for patients with advanced, unresectable colorectal cancer. The aim of this review is to critically focus on updated scientific data that medical oncologists need to interpret to make the most appropriate evidence-based choice among many possible treatment options.

Aprile G, Lutrino SE, Ferrari L, Casagrande M, Bonotto M, Ongaro E, Puglisi F. Evidence-based appraisal of the upfront treatment for unresectable metastatic colorectal cancer patients. *World J Gastroenterol* 2013; 19(46): 8474-8488 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i46/8474.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i46.8474

WHICH REASONING DOES LIE BENEATH THE CHOICE OF A FIRST-LINE TREATMENT?

Colorectal cancer (CRC) is currently the second most common cancer in Europe, with nearly 450000 new cases and approximately 215000 deaths occurred in 2012[1]. Half of those patients are either initially diagnosed at an advanced or metastatic stage or later develop distant metastases, and have a 5-year survival rate of 5%-10%[2]. While chemotherapy following resection of liver or lung

metastases has been reported to increase the chance of cure in selected patients, palliative systemic treatments may at least produce survival benefits for those presenting with diffuse unresectable disease. Over the last two decades, the median survival of patients with metastatic CRC has progressively improved, approaching 30 mo in recent reports. Notably, not only the widespread use of all available active agents (including 4 different chemotherapy drugs and 5 biologics) has shaped this clinical success, but also more patients have profited enhanced quality of life while receiving modified or less intensive maintenance treatments or while enjoying chemotherapy-free intervals. In fact, a smoother, more plastic concept embracing a "comprehensive treatment strategy" has substituted the rigid classical sequence of following structured treatment lines in the continuum of care. Notwithstanding those significant advances, the treatment landscape for unresectable advanced CRC has become increasingly complex. For all those incurable patients, mainstay of the treatment is to maximise survival while minimizing toxicities and maintaining optimal quality of life. The availability of more therapeutic options, however, has generated intricate algorithms of treatment decision-making and medical oncologists are often overwhelmed by a large number of trials providing unclear or conflicting results.

Unquestionably, when deciding the delivery of an optimally personalized treatment sequence, the ultimate treatment goal, outcome data from randomized clinical trials, different regimen-related toxicity profiles, molecular status of the disease, and patients' willingness should all be considered. However, while recent guidelines suggest to combine chemotherapy with targeted agents for the vast majority of those aged less than 75 years[3], it is much less clear which patients deserve a higher treatment intensity and which is the best biologic to use upfront for CRC patients with KRAS wild-type disease[4]. Moreover, it should be acknowledged that the proportion of patients receiving therapy diminishes with subsequent lines and that efficacy results are the greatest in untreated patients and usually reduce along with treatment course because of a growing degree of chemoresistance. The foundation of the upfront treatment is, therefore, crucial: in first-line setting the highest number of patients may benefit therapies with the highest response rates and the longest median progression-free survival (PFS). Moreover, there is still a chance for unexpected resection and even cure, and for all those who will not be cured, first-line therapy may impact on overall survival (OS).

Actually, whenever discussing with a previously untreated patient the different first-line treatment options, some clinical considerations should be made: (1) How long will the patient survive and how long will the patient benefit from first-line treatment? (2) Does the patient need (and agree on) an aggressive strategy? (3) Will a deeper knowledge of tumor molecular biology aid in the decision-making process? (4) May the patient benefit from maintaining an antiangiogenic strategy across treatment lines? and (5) Has the first-line choice potential impact on further treatment lines?

In addition, if the patients has previously received adjuvant chemotherapy (indeed, approximately 30% of metastatic CRC patients had), other questions arise: (1) How long have the patient lived without evidence of disease? (in other words, how long did the disease-free interval last?) and (2) May previous adjuvant treatments condition the first-line treatment choice?

Reporting as a springboard for discussion results from key randomized clinical trials (Table 1), aim of this viewpoint is to help clinicians making an evidence-based decision when choosing among possible first-line treatments for their medically-fit advanced unresectable CRC patients.

WHEN TO TREAT PATIENTS WITH HIGHER INTENSITY? SEARCHING FOR THE OPTIMAL FINE-TUNING

The idea of combining all available drugs upfront with the aim to hit and immediately kill as many cancer cells as possible is certainly not new. In CRC, the combination of 5-fluorouracil, oxaliplatin, and irinotecan (FOLFOXIRI) was initially compared to 5-fluorouracil and irinotecan (FOLFIRI) in two independent studies[5,6]. Results from the phase Ⅲ randomized Italian trial showed significant advantage for the triplet in terms of RR (66% *vs* 41%, $P = 0.0002$), PFS (9.8 mo *vs* 6.9 mo, HR = 0.63), OS (22.6 mo *vs* 16.7 mo, HR = 0.70), and secondary resections for those with liver-limited disease (36% *vs* 12%, $P = 0.01$), thus presenting such an intensive upfront regimen among the potential choices to be used when a significant tumor shrinkage is needed. Oppositely, although based on an encouraging preclinical[7] and clinical[8] background, final results of combining doublet chemotherapy with both bevacizumab and Epidermal Growth Factor Receptor (EGFR)-inhibitors were vastly disappointing[9,10]. Overall, both the randomized phase Ⅲ CAIRO2 and PACCE studies showed significantly reduced PFS outcome results and increased toxicity profiles for the 4-drugs combination when compared to chemotherapy plus bevacizumab alone. The reasons for the unforeseen antagonism between the two biologic agents when combined with chemotherapy are still uncertain[11]. The issue regarding how much intense the chemotherapy backbone should be remains critical also in the era of targeted agents. Two randomized trials, phase Ⅲ TRIBE[12] and phase Ⅱ OLIVIA[13], investigated the combination of the FOLFOXIRI based-regimen with the antiangiogenic bevacizumab. In the first trial, 508 advanced CRC patients received upfront FOLFIRI or FOLFOXIRI plus bevacizumab. Patients in the experimental arm achieved a significantly longer PFS (12.1 mo *vs* 9.7 mo; HR = 0.77, 95%CI: 0.64-0.93, $P = 0.006$). The triplet also provided a significant increase in RR (65% *vs* 53%, $P = 0.006$), but not in radical resection rate (15% *vs* 12%, $P = 0.327$). Neverthe-

Table 1 Outcome results of major randomized phase Ⅲ trials in the first-line setting in metastatic colorectal cancer patients

Ref.	Regimen	*n*	Previous adjuvant treatment	ORR	Median PFS (mo)	Median OS (mo)	Post-study therapy
Hurwitz *et al*[80] Tebbutt *et al*[119]	IFL	411	28%	34.8%	6.2	15.6	50%
Hurwitz *et al*[80]	IFL+bevacizumab	402	24%	44.8%	10.6	20.3	50%
Cunningham *et al*[118]	Capecitabine	140	18.6%	10%	5.1	16.8	37%
	Capecitabine + bevacizumab	140	32.1%	19%	9.1	20.7	37%
Saltz *et al*[8]	XELOX/FOLFOX	701	25%[1]	38%	8	19.9	53%
	XELOX/FOLFOX + bevacizumab	699	24%[1]	38%	9.4	21.3	46%
Heinemann *et al*[81]	FOLFIRI + cetuximab	297	22.1%	62%	10	28.7	65.7%
	FOLFIRI + bevacizumab	295	18.9%	58%	10.3	25	61.7%
	Capecitabine	156	22%	30.3%	5.7	18.9	68%
Tebbutt *et al*[119]	Capecitabine + bevacizumab	157	28%	38.1%	8.5	18.9	62%
	Capecitabine + bevacizumab + MMC	158	16%	45.9%	8.4	16.4	61%
Falcone *et al*[12]	FOLFOXIRI + bevacizumab	252	12%	65%	12	31	NA[3]
	FOLFIRI + bevacizumab	256	12%	53%	9.7	25.8	NA[3]
Van Cutsem *et al*[82]	FOLFIRI	599	18.9%	39.7%	8.4	20	71.7%
	FOLFIRI + cetuximab	599	17.4%	57.3%	9.9	23.5	66%
Maughan *et al*[91]	XELOX/FOLFOX[2]	815	25%[1]	57%	8.6	17	62%
	XELOX/FOLFOX + cetuximab	815	25%[1]	64%	8.6	17.9	56%
Tveit *et al*[120]	FLOX	185	8%[1]	41%	7.9	20.4	73.5%
	FLOX + cetuximab	194	9%[1]	49%	8.3	19.7	75.8%
	FLOX intermittently + cetuximab	187	10%[1]	47%	7.3	20.3	64.2%
Douillard *et al*[90]	FOLFOX4	590	15%[1]	48%	8	19.7	63%
	FOLFOX4 + panitumumab	593	16.1%[1]	55%	9.6	23.9	53%
Schmoll *et al*[89]	FOLFOX + bevacizumab	713	19%	47.3%	10.3	21.3	23.8%
	FOLFOX + cediranib	709	17%	46.3%	9.9	22.8	28.2%
Díaz-Rubio *et al*[60]	XELOX + bevacizumab	239	13%[1]	47%	10.4	23.2	72%
	XELOX + bevacizumab→bevacizumab	241	17%[1]	49%	9.7	20	74%

[1]No previous oxaliplatin-based treatment allowed; [2]Both Arm A (continuously) and Arm B (intermittently) have been considered; [3]Data will be available in 2014. ORR: Overall response rate; PFS: Progression-free survival; OS: Overall survival; IFL: Irinotecan, fluorouracil, leucovorin therapy; FOLFIRI: 5-fluorouracil and irinotecan; XELOX: Capecitabine/oxaliplatin.

less, the study population was unselected for conversion to surgical resectability, since only 20% of randomized patients had liver-limited disease. Preliminary data showed a trend toward improved OS in the FOLFOXIRI plus bevacizumab arm (31.0 mo *vs* 25.8 mo; HR = 0.83, 95%CI: 0.66-1.05). Phase Ⅱ OLIVIA trial allocated 80 advanced CRC patients with liver-only unresectable metastases to receive 5-fluorouracil and oxaliplatin (FOLFOX) or FOLFOXIRI plus bevacizumab. Overall resection rate, the primary endpoint, was numerically higher in the FOLFOXIRI plus bevacizumab arm (61.0% *vs* 48.7%, $P = 0.27$). The more intensive regimen provided both a higher RR (80.5% *vs* 61.5%, $P = 0.061$) and radical (R0) resection rate (48.8% *vs* 23.1%, $P = 0.017$), with longer PFS (18.8 mo *vs* 12.0 mo, $P = 0.0002$). Moreover, retrospective data suggest that the addition of bevacizumab to the FOLFOXIRI regimen does not impact on liver toxicity while enhancing the rate of pathologic response and tumor necrosis[14].

The combination of FOLFOXIRI with EGFR-inhibitors showed also interesting results in a phase Ⅱ trial, but a formal phase Ⅲ comparison of the added benefit of cetuximab or panitumumab to the triplet regimen is currently lacking. In the TRIP study, 37 highly molecularly selected patients (concomitant wild-type status for KRAS, BRAF, NRAS, and HRAS) received FOLFOXIRI plus panitumumab with a reported RR of 89%. Forty-three percent of them underwent secondary surgery of metastases, and R0 resection was achieved in 13 cases (35%). After a median follow-up of 17.7 mo, median PFS was 11.3 mo[15]. Another phase Ⅱ study enrolled 43 CRC patients with unresectable liver metastases to receive cetuximab plus chronomodulated irinotecan, 5-fluorouracil, leucovorin and oxaliplatin as neoadjuvant chemotherapy[16]. After a median number of 6 cycles, RR was noted in 79% of patients, and median OS was of 37 mo.

Based on available results, when should we opt for a very intensive treatment? The use of triplet plus bevacizumab could be considered a possible treatment option for those who parallel the trial's inclusion criteria (*i.e.*, unresectable, metastatic disease, age < 75 years; optimal ECOG PS, no major comorbidities), but this appears to be a much more intriguing and logical option for patients with symptomatic, bulky or aggressive disease or when conversion from unresectable to resectable status is deemed possible (liver-limited unresectable metastases). In the first circumstance, patients may benefit from a fast disease shrinkage that while reducing the tumor burden may better control cancer-related symptoms or avoid their occurrence. In the second condition, the advantage of using this highly active combination is that it may exert its effect in few cycles, avoiding a sustained exposure to chemotherapy that might potentially increase liver toxicity just before hepatic surgery. Although phase Ⅱ studies results are promising, the use of a triplet regimen combined with EGFR-inhibitors outside of a clinical trial

should be currently discouraged, even in patients with optimal molecular selection. In order to ameliorate the tolerability, the intensification of the upfront therapy in never resectable patients usually requires to plan a short initial treatment period (induction phase) followed by a less intensive treatment (maintenance phase). To avoid excessive toxicity in a palliative setting, the strength of such an induction treatment should last no longer than 8 cycles. After that, patients are usually switched to an appropriate, more tolerable, maintenance regimen that may be continued for a long period. Ongoing studies are clarifying the role of the maintenance therapy and expounding which are the optimal agents to be used. Potential drawbacks of an intensive treatment include higher toxicity and more limited rescue options once the tumor has become resistant.

WHICH BIOLOGIC SHOULD BE PREFERRED IN THE UPFRONT TREATMENT OF KRAS WILD-TYPE CRC PATIENTS?

Although the predictive role of G13D mutation still remains a matter of discussion[17-19], having a KRAS mutation in codon 12 or 13 is a universally accepted marker for EGFR-inhibitor inefficacy[20,21]. Other germline mutations in *RAS* or *BRAF* genes also seem to predict unfavourable results[22,23], and acquired secondary mutations may cause resistance to EGFR-inhibitors[24-26]. Moreover, retrospective data confirmed that using a more adequate technique RAS or BRAF mutations were found in approximately 20% of cancers initially classified as wild-type[20], and this might help in refining the target population[27,28]. Current molecular selection has a negative predictive value, but it does not help in the clinical-decision process for patients with wild-type CRC. Actually, which targeted agent should be combined to first-line chemotherapy in KRAS wild-type patients is one of the hot-topics in colorectal oncology. Up today, the choice was essentially based on cross-trial comparisons and on meta-analyses estimating the magnitude of benefit provided by each targeted agent[29,30]. While EGFR-inhibitors were considered powerful shrinking agents, bevacizumab was preferred for its ability to delay tumor progression. FIRE-3, the first phase Ⅲ randomized trial to provide results on the head-to-head comparison, randomized 592 KRAS wild-type CRC patients to upfront FOLFIRI plus either cetuximab or bevacizumab, with the aim to detect a difference of 12% in RR induced by FOLFIRI plus cetuximab (62%) compared to FOLFIRI plus bevacizumab (50%)[31]. Though unusual for a randomized phase Ⅲ trial, RR was chosen as the primary endpoint of the study. Because of a higher than expected treatment activity reported for patients exposed to bevacizumab, RR resulted similar between treatment arms (62% in the FOLFIRI plus cetuximab arm *vs* 58% in the FOLFIRI plus bevacizumab arm, OR = 1.18, $P = 0.18$) and no differences in PFS were documented (HR = 1.06; 95%CI: 0.88-1.26, $P = 0.54$). Of note, in the cohort of patients assessable for response ($n = 526$, 89%), encompassing all those who had received a minimum of 3 cycles and had performed at least a CT-scan evaluation following baseline, RR was significantly higher in favour of cetuximab-containing arm (72.2% *vs* 63.1%, OR = 1.52, $P = 0.017$). Although, no significant differences in median PFS were reported (10 mo *vs* 10.3 mo, HR = 1.03; 95%CI: 0.88-1.26), a clinically meaningful 3.7-month median advantage in OS was evidenced in favour of the cetuximab arm (28.7 mo *vs* 25 mo, HR = 0.77; 95%CI: 0.62-0.96), confirmed in all exploratory subgroups analysed. Disparities in subsequent treatment lines may hardly explain this unforeseen survival difference, being the proportion of patients who crossed over or received treatment beyond progression similar between treatment arms (65.7% in the cetuximab arm *vs* 61.7% in the bevacizumab arm, $P = 0.34$). Oppositely, the association of both early tumor shrinkage (at least 20% decrease in the sum of the longest diameter compared with baseline at week 8) and the deepness of response (percentage of tumor shrinkage observed at the smallest tumor size compared to baseline) to EGFR-inhibitors with the post-progression survival were advocated as possible reasons for success[32]. According to this theoretical model, the higher tumour shrinkage may result in a lower tumour load, as per RECIST, at the time of disease progression so that the benefit achieved in terms of deepness of response may influence the following history of patients' disease. Likewise, a significant correlation of the early objective tumor response (EOTR) with survival was demonstrated by an individual patient data meta-analysis of 15 randomized first-line trials enrolling approximately 12000 patients from the ARCAD database[33]. In the analysis, median PFS and median OS were consistently longer in patients with an EOTR at 6, 8 or 12 wk compared to those without. Overall, these results support the hypothesis that the advantage in terms of activity of an intensive upfront regimen may translate into a significant survival gain regardless the opportunity to achieve secondary resections. While a confirmatory correlation analysis is being conducted in FIRE-3 trial, outcome results from a larger intergroup phase Ⅲ trial (CALGB 80405, NCT00265850) that aims to compare upfront chemotherapy with bevacizumab or cetuximab in over 1200 metastatic CRC patients are awaited. Differently from FIRE-3, OS is the primary endpoint of the CALGB and SWOG cooperative groups trial.

To simultaneously explore the head-to-head comparison and the treatment strategy, the GERCOR is sponsoring the phase Ⅲ STRATEGIC-1 trial[34] that is designed to provide information on the optimal treatment sequence, with two different strategies each including all the currently available agents (oxaliplatin, irinotecan, fluoropyrimidines, bevacizumab, and EGFR-inhibitors), but in a different order. With disease control rate of the full strategy as the primary endpoint, nearly 500 patients with unresectable wild-type KRAS metastatic CRC will be

randomized to FOLFIRI-cetuximab, followed by an oxaliplatin-based chemotherapy with bevacizumab (Strategy A) or OPTIMOX-bevacizumab, followed by irinotecan-based chemotherapy with bevacizumab, followed by an EGFR-inhibitor with or without irinotecan (Strategy B). The study is starting soon the target recruitment.

TOWARD A BETTER MOLECULAR SELECTION? BROADENING CRC BIOLOGIC KNOWLEDGE BEYOND KRAS

Since the acknowledgment that CRC is a highly heterogeneous disease with regards to clinical evolution and response to treatments and the fact that it may change over time or evolve under treatment pressure[35], a more profound molecular knowledge of this cancer has been promoted[36]. Actually, a deeper understanding of the disease pathobiology and its molecular underpinnings allow clinicians to take advantage of a more detailed disease classification[37] and more robust information on predictive and prognostic biomarkers as well as resistance bioindicators for both antiangiogenic[38] and EGFR-inhibitors[39]. Whether serial tumor biopsies and repeated mutation testing may be useful to better capture the CRC heterogeneity and to systemically track its genomic evolution is a matter of debate[40,41], but the application of innovative, low-invasive techniques may find acceptance from both scientific and ethical standpoints[42,43]. Specifically focusing on the treatment tailoring, the landscape has rapidly evolved beyond KRAS codon 12 and 13 mutational status[44]. For example, rare mutation occurring in other KRAS codons, such as mutation in codons 61 or 146, may result in reduced EGFR-inhibitor efficacy[22]. As well, V600E BRAF mutations occurring in approximately 10% of all KRAS wild-type CRC tumors[45] or more rare KRAS amplifications[46] seem to limit the benefit from EGFR-inhibitors[47-49]. However, while there is total agreement on its negative prognostic value, the negative predictive role of BRAF mutations with regards to EGFR-inhibitor therapy is not universally accepted[50-52] and loss of PTEN expression or activity[53,54] have also been associated to inferior benefit from EGFR-inhibitors, but the small sample size of the cohort analysed linked to the relatively rare events prevent to draw strong definitive conclusions.

Importantly, the use of EGFR-inhibitors in the clinical practice should be based on a deep molecular analysis with further refinement of tumor-specific genetic markers in order to simultaneously allow: (1) identification of a wider patient population that does not benefit from the target treatment or may have detrimental effect; and (2) selection of patients who may achieve a maximized survival improvement. A prospective-retrospective analyses of phase Ⅲ PRIME trial[55] that randomized 1083 patients to upfront FOLFOX plus or minus panitumumaband a preplanned analysis of phase Ⅱ PEAK study that assigned in first-line 285 patients to FOLFOX plus either bevacizumab or panitumumab[56] consistently show that patients harbouring rare KRAS mutations in exon 3 (codons 59/61) and 4 (codons 117/146), or NRAS mutations in exon 2 (codons 12/13), 3 (codons 59/61), and 4 (codons 117/146) may not benefit from the EGFR-inhibitor. In the first analysis, patients without RAS mutations had a 2.2 mo median advantage in median PFS (10.1 mo *vs* 7.9 mo, HR = 0.72, 95%CI: 0.58-0.9, $P = 0.004$), and a 5.8 median advantage in OS (26 mo *vs* 20.2 mo, HR = 0.78, 95%CI: 0.62-0.99, $P = 0.04$). Impressively, patients with no RAS or BRAF mutations ($n = 446$) derived a 7.6 median survival benefit (28.3 mo *vs* 20.9 mo, HR = 0.74, 95%CI: 0.57-0.96, $P = 0.02$) if exposed to FOLFOX and panitumumab in first-line. An exploratory biomarker tumor analysis[57] of patients enrolled in the panitumumab *vs* BSC randomized phase Ⅲ study[58] reported similar results. Importantly, the addition of panitumumab to first-line FOLFOX might be even detrimental in patients with less common RAS mutations and should be cautiously avoided. On the basis of these data, marketing authorization for panitumumab has been amended, including the analysis of NRAS status before prescription, and restraining its use to RAS wild-type CRC patients. Since it has been highlighted how a more detailed molecular profile may impact on the evidence-based decision making process, a more accurate selection of candidates to upfront EGFR-inhibitors is warranted. Results of a similar deeper molecular analysis in patients exposed to upfront cetuximab or bevacizumab combined with FOLFIRI in the FIRE-3 trial will be soon presented.

ANGIOGENIC INHIBITORS UPFRONT AND IN THE FOLLOWING TREATMENT LINES? THE ISSUE OF MAINTENANCE AND TREATMENT BEYOND PROGRESSION

The choice of an upfront bevacizumab-based combination is considered a widely accepted standard treatment option for the majority of advanced CRC patients. Although supported by limited evidence, to continue the angiogenic inhibitor until disease progression is not uncommon in the clinical practice, especially for those patients who partially or entirely withhold the associated chemotherapy because of toxicity or towering cumulative doses of oxaliplatin[59]. Actually, results of randomized trials such as MACRO[60], DREAM[61], and COIN-B[62] suggest to continue bevacizumab as maintenance therapy until disease progression. In the MACRO trial, 480 CRC patients were randomly assigned to receive six cycles of bevacizumab, capecitabine, and oxaliplatin followed by bevacizumab either alone or combined with the same chemotherapy regimen until progression. A slightly longer median PFS was reported in the combination arm (10.4 mo *vs* 9.7 mo, HR = 1.1, $P = 0.38$), although burdened by a higher rate of severe sensory neuropathy (26% *vs* 8%, $P = 0.0001$) and HFS (13% *vs* 7%, $P = 0.03$). The primary analysis of DREAM demonstrated that a maintenance

therapy with bevacizumab and erlotinib may significantly prolong median PFS (10.2 mo *vs* 9.3 mo, HR = 0.76; 95%CI: 0.61-0.94, *P* = 0.009) but not median OS (28.5 mo *vs* 27.0 mo, HR = 0.89; 95%CI: 0.7-1.12, *P* = 0.31) after a first-line bevacizumab-based induction therapy[63]. The additive value of erlotinib to bevacizumab in this setting is however unconfirmed[64]. Yet, the issue regarding the role of bevacizumab in the maintenance phase was not formally addressed until recently. SAKK 41/06[65] and CAIRO-3[66] phase Ⅲ trials compared observation to a maintenance strategy following an induction phase of chemotherapy plus bevacizumab. In the non-inferiority Swiss study, 262 CRC patients without disease progression at 4-6 mo since treatment start were randomized to continue on single-agent bevacizumab until disease progression or observation. Even though median PFS (+ 1.2 mo) and OS (+ 3.3 mo) were both longer for patients who continued on bevacizumab, the trial formally failed to meet its primary endpoint, since the median time to progression did not differ sufficiently between treatment arms (17.9 wk *vs* 12.6 wk; HR = 0.74; 95%CI: 0.57-0.94, *P* = 0.47; with a non-inferiority limit for HR = 0.727). In CAIRO-3 trial, patients without disease progression after 6 cycles of capecitabine, oxaliplatin (CAPOX regimen) and bevacizumab were randomized to observation or continuing with capecitabine and bevacizumab. Upon the first disease progression, CAPOX plus bevacizumab was reintroduced and maintained until the second evidence of progression. The primary endpoint was the PFS2, defined as the time from randomization to progression upon treatment re-introduction. Patients in the maintenance arm achieved a significantly longer PFS2 (11.8 mo *vs* 10.5 mo, HR = 0.81; 95%CI: 0.67-0.98, *P* = 0.028), PFS (8.5 mo *vs* 4.1 mo, HR = 0.44; 95%CI: 0.36-0.53, *P* < 0.00001) and a non-significant advantage in OS (21.7 mo *vs* 18.2 mo, HR = 0.87; 95%CI: 0.71-1.06, *P* = 0.156), that became significant in the adjusted analysis (HR = 0.80). AIO KRK0207, a phase Ⅲ randomized trial comparing observation to maintenance with either bevacizumab alone or bevacizumab plus capecitabine, will clarify if a maintenance treatment, instead of a full holiday period, is actually needed for all patients. In conclusion, while reasonable, safe, and clinically feasible, whether a maintenance therapy is needed for all patients is still an open question.

The role of cetuximab in the maintenance therapy is also being investigated. The two-arm phase Ⅱ COIN-B study randomized 169 patients with unresectable KRAS wild-type CRC to intermittent chemotherapy plus continuous or intermittent cetuximab as first-line treatment. Continuous cetuximab was associated with a longer failure free survival (FFS), chemotherapy-free interval (3.7 mo *vs* 5.1 mo) and time to progression (20.1 mo *vs* 18.4 mo). Median FFS was 12.0 and 13.7 mo, respectively[62]. The phase Ⅲ Macbeth trial (EUDRACT 2011-000840-70) is an ongoing multicenter, randomized, open-label study designed to evaluate the efficacy and safety of eight cycles of FOLFOXIRI plus cetuximab followed by maintenance with cetuximab or bevacizumab as first-line treatment for unresectable KRAS wild-type metastatic CRC patients.

Another point of discussion is the use of antiangiogenics beyond disease progression. Data from retrospective registries such as BRITE[67] or ARIES[68] suggested a survival benefit with the use of bevacizumab beyond disease progression. More recently, the randomized phase Ⅲ ML18147 trial prospectively tested the efficacy of maintaining bevacizumab beyond disease progression[69]. After the failure of a bevacizumab-containing first-line treatment, 820 patients were randomized to receive a different second-line chemotherapy with or without bevacizumab. Those that continued on the antiangiogenic agent reported significantly longer OS (11.2 mo *vs* 9.8 mo; HR = 0.81; 95%CI: 0.69-0.94, *P* = 0.0062) and PFS (5.7 mo *vs* 4.1 mo, HR = 0.68; 95%CI: 0.59-0.78, *P* < 0.0001). Toxicity profiles were similar between the two arms, although more bleedings (2% *vs* 1%), venous thromboembolic events (5% *vs* 3%), and gastrointestinal perforations (2% *vs* < 1%) were noted among those receiving bevacizumab. In the phase Ⅲ BEBYP trial[70], 184 patients who had failed a bevacizumab-based first-line treatment were randomized to receive second-line chemotherapy with or without bevacizumab. The trial was stopped early, as soon as the positive results of the ML18147 were diffused. Performance status (ECOG PS 0 *vs* 1-2), length of the chemotherapy-free interval (< or > 3 mo), and type of second-line chemotherapy were considered as stratification factors. Two thirds of the patients received oxaliplatin-based combinations in both treatment arms. After a median follow-up of 22 mo, the results confirmed the benefit in PFS (6.8 mo *vs* 5 mo, HR = 0.72; 95%CI: 0.54-0.97, *P* = 0.029) for those maintained on bevacizumab, while OS data are still immature to be analyzed.

Indirect evidence supports how CRC patients may benefit from further angiogenic treatments after disease progression while on bevacizumab. The phase Ⅲ VELOUR trial showed the efficacy of aflibercept (a fusion protein with high affinity to all VEGF-A isoforms, VEGF-B, PlGF-1, and PIGF-2) in combination with FOLFIRI in 1,266 CRC patients who had failed a first-line oxaliplatin-based therapy[71]. Both median OS (13.5 mo *vs* 12.06 mo, HR = 0.817; 95%CI: 0.71-0.94, *P* = 0.0032) and PFS (6.9 mo *vs* 4.67 mo, HR = 0.76) were significantly longer in those who received FOLFIRI and aflibercept. Importantly, prior exposition to antiangiogenics did not reduced the outcome effect. Actually, a similar benefit in PFS (6.7 mo *vs* 3.9 mo, HR = 0.66; 95%CI: 0.51-0.85) and OS (12.5 mo *vs* 11.7 mo, HR = 0.86; 95%CI: 0.67-1.10) was reported for the use of aflibercept in those who had received bevacizumab as part of their upfront treatment (approximately 28% in both treatment arms). Regorafenib is another agent with broad antiangiogenic properties[72]. In the CORRECT trial, 760 chemorefractory CRC patients were randomized 2:1 to regorafenib (160 mg daily in a 3-wk-on, 1-week-off schedule) or placebo[73]. All patients had previously re-

ceived bevacizumab. Median OS was 6.4 mo in the regorafenib group *vs* 5.0 mo in the placebo group (HR = 0.77; 95%CI: 0.64-0.94).

Large, international efforts have tried to define who are the patients more likely to benefit from the antiangiogenic strategy. Unfortunately, given the complexity of cancer-related angiogenesis, conflicting results have been reported both at molecular[74] or clinical levels[75,76]. The prospective validation of other single predictive biomarkers such as baseline LDH value[75], number of circulating endothelial cells[77], or level of miRNA[78] are still pending, but will unlikely succeed.

WILL THE FIRST-LINE CHOICE IMPACT ON FOLLOWING TREATMENT LINES?

If and how the first-line therapy may influence further treatment is a matter of debate at many levels (molecular, clinical, regulatory). Nevertheless, how oncologists decide the sequence of treatment to use should be always based on a solid mainstay. The following reasoning is founded on a critical analysis of major phase Ⅲ randomized studies.

Accordingly to the results of a pivotal phase Ⅲ trial that compared FOLFOX6 followed by FOLFIRI to FOLFIRI followed by FOLFOX6 and showed similar outcomes regardless of the treatment sequence[79], the backbone treatment used after first disease progression of disease is currently based on a crossover from an irinotecan- to an oxaliplatin-based regimen or vice-versa. In that trial, 220 patients were randomized to receive initially either FOLFIRI or FOLFOX6 and to switch to the other regimen at disease progression. Neither first-line RR (56% *vs* 54%), nor first-line median PFS (8.5 mo *vs* 8 mo, P = 0.26), nor median OS (21.5 mo *vs* 20.6 mo, P = 0.99) were statistically different between treatment arms.

Ten years after the widespread use of biologics has begun in the clinical practice, the scenario has become much more complicated, particularly in patients with KRAS wild-type tumors that may benefit from a scope of different treatments. The initial choice of the upfront chemotherapy regimen, however, retains its value.

When opting for an irinotecan-based first-line regimen, either bevacizumab[80] or cetuximab[81,82] could be used as optimal biologic partners. Either way the patient is started, survival results of the ECOG E3200 phase Ⅲ trial[83] would suggest to use FOLFOX plus bevacizumab as second-line treatment after an irinotecan-based first-line failure. Later on, following on the treatment route, the choice of third-line may become critical. In this setting, while strong data support the use of EGFR-inhibitors either alone[84,58] or combined to irinotecan[85],evidence suggesting potential benefit from retreating patients with EGFR-inhibitors is more shaggy[86,87] or under investigation[88]. Regorafenib, indeed, would be an appropriate choice for all highly pretreated patients[73]. Consequently, the treatment algorithm would offer 4 potential lines of treatment if the patient receive upfront an irinotecan-based chemotherapy plus bevacizumab, but one treatment line would be lost if the patient starts with an irinotecan-based therapy plus cetuximab. This hypothetical reasoning may be revised (and even reversed) if the outcome results of CALGB 80405 trial will confirm the unexpected 3.7-mo median survival advantage reported in FIRE-3 for KRAS wild-type CRC patients receiving FOLFIRI and cetuximab in first-line.

When opting for a first-line treatment including oxaliplatin, antiangiogenic drugs[60,89] or EGFR-inhibitor[90,91] may be used in combination, although the upfront use of bevacizumab seems to be preferable because it may better fit in the maintenance strategy[92,93] for its convenience and safety when combined to capecitabine[94]. Moreover, the upfront combination of oxaliplatin with an EGFR-inhibitor requires more detailed molecular biology data (see paragraph 4) and increased watchfulness if using an oral fluoropyrimidine[91]. At disease progression, many reasons strongly support the choice of switching to an irinotecan-based regimen, including the potential cumulative neurotoxicity of prolonged oxaliplatin use. Since in second-line setting many alternative options exist, to establish which is the optimal biologic to be delivered is challenging and depends on the previous use of targeted agents. A number of second-line randomized trials have investigated the role of biological agents in the treatment of CRC patients not previously exposed to EGFR-inhibitors. Tested agents included bevacizumab[69], aflibercept[71], cetuximab[95], or panitumumab[96,97]. Of note, in all those trials patients may have been upfront treated with bevacizumab, but the proportion of those who did receive the angiogenic inhibitors in first-line vastly varied, ranging from 2%[97] to 100%[69]. Results of ML18147 and VELOUR have been already discussed (see before). In the phase Ⅲ EPIC study[92], 1298 patients who had prior failed a first-line oxaliplatin-based regimen, were randomized to receive irinotecan plus cetuximab or irinotecan alone. The addition of cetuximab to irinotecan resulted in a significant improvement of PFS (4.0 mo *vs* 2.6 mo, HR = 0.69; 95%CI: 0.617-0.776, P < 0.0001), but no OS advantage was reported (10.7 mo *vs* 10.0 mo, HR = 0.97). Panitumumab was tested in another randomized phase Ⅲ trial, comparing in 1,186 pretreated metastatic CRC patients, the addition of panitumumab itself to FOLFIRI, to placebo. A significant improvement in PFS was observed (5.9 mo *vs* 3.9 mo, HR = 0.73; 95%CI: 0.59-0.90, P = 0.004), with a trend for longer OS (14.5 mo *vs* 12.5 mo, HR = 0.85; 95%CI: 0.70-1.04, P = 0.12). Similarly, the PICCOLO study[97] reported higher RR (34% *vs* 12%, P < 0.001), longer PFS (HR = 0.78; 95%CI: 0.64-0.95, P = 0.015), but no survival advantage (10.9 mo *vs* 10.4 mo; HR = 1.01; 95%CI: 0.83-1.23, P = 0.91) for the use of panitumumab and irinotecan-based chemotherapy compared to irinotecan alone. If the upfront biologic was the EGFR-inhibitor, less options are permitted (see point A). Again, regorafenib may be considered as salvage treatment for all pretreated patients. As discussed before, if the patient is started with a EGFR-inhibitor, the number

of therapeutic options seems narrowed.

CHOOSING A FIRST-LINE TREATMENT FOR CRC PATIENTS WHO HAVE FAILED ADJUVANT OXALIPLATIN - IS THERE ANY DIFFERENCE?

Since approximately 50% of stage Ⅲ and 20% of stage Ⅱ CRC patients do eventually recur, one third of patients present with metachronous metastatic disease, which is currently defined as more than 1 year between the occurrence of the primitive tumor and metastasis. Not surprisingly, a significant proportion of those patients may have already received an oxaliplatin-based chemotherapy, a universally confirmed standard regimen in the adjuvant setting[98-100]. Indeed, patients enrolled in first-line phase Ⅲ randomized trials which had already been exposed to adjuvant chemotherapy ranged from 8% to 32% (Table 1). However, having received a previous treatment with oxaliplatin was sometimes included among the exclusion criteria, and even when it was permitted, how many of those pretreated patients had actually received an oxaliplatin-based regimen was rarely specified in the publication.

To fully understand the importance of this point, some data should be further discussed. The analysis of over 20000 CRC patients included in the ACCENT database showed that the risk of recurrence peaks between 18 and 24 mo after radical surgery, and then decreases over time[101]. Most patients who recur, therefore, develop metastatic disease within 18 mo since the end of postoperative chemotherapy.

The use of oxaliplatin is burdened by the frequent occurrence of chronic peripheral sensory neuropathy[102,103], a dose-dependent disturbing toxicity characterized by dysesthesia and distal paresthesia, that often negatively impacts on patients' quality of life[104]. In addition, acute neuropathy (oral-facial and peripheral), which in some cases is induced or exacerbated by exposure to cold, was also reported. This neurological side-effect, quite unusual in the initial chemotherapy cycles, frequently appears during the treatment course as long as the cumulative dose of oxaliplatin increases.

The vast majority of the patients enrolled in randomized clinical trials that tested oxaliplatin in the adjuvant setting developed peripheral sensory neuropathy. In MOSAIC trial, any grade peripheral neurotoxicity was observed in 92% of patients, while grade 2 (moderate) or grade 3 (severe) was reported in 44%. Often, however, the symptoms ameliorated or resolved over time: one and four years after treatment, 30% and 15% of patients had minimal residual toxicity, respectively. In NSABP C-07 trial, grade 3-4 peripheral neuropathy was reported in 8.4% of patients. At 1 year from random assignment, the rate of severe neurotoxicity was 0.6%. The inferior rate of neurotoxicity may be due to the lower cumulative dose of oxaliplatin in NSABP C-07 (9 planned doses of 85 mg/m^2) compared to MOSAIC (12 planned doses of 85 mg/m^2).

In NO16968 study, any grade peripheral neuropathy occurred in 78% of patients exposed to oxaliplatin, and grade 3-4 in 11%. At the end of adjuvant treatment, residual neurotoxicity was still present in 68% of patients.

Toxicity data were confirmed in another randomized trial that tested the efficacy of bevacizumab combined to oxaliplatin-based chemotherapy in the adjuvant setting[105]. Grade 2 or grade 3 sensory neuropathy was reported in 43.7% of patients treated with FOLFOX6 and in 48.9 % of those treated with FOLFOX6 + bevacizumab, with the delivery of similar median doses of oxaliplatin. Notably, about 10%-20% of patients developed severe neurotoxicity after cumulative oxaliplatin dose of 750-850 mg/m^2[106].

Recently, a number of studies reported on a long-lasting oxaliplatin-induced peripheral neurotoxicity[107,108]. Those studies showed that a not-negligible proportion of patients (5%-15%) still suffer from chronic neurotoxicity many years after treatment end, and refer troublesome numbness or tingling of hands and feet. Than, it is conceivable that a proportion of oxaliplatin-exposed patients may still have neurological symptoms at the time of recurrence. In order to prevent or reduce the incidence and intensity of this toxicity in the adjuvant setting, several strategies are being studied, including a reduced exposition to oxaliplatin[109] or the potential use of neuroprotectants such as glutathione[110], oxcarbazepine[111], or venlafaxine[112], but no preventive treatment has been recognized as a standard. Moreover, retrospective studies suggested that the *iv* supplementation with calcium and magnesium may be useful[113]. However, a randomized phase Ⅲ trial enrolling 362 radically resected CRC patients with no pre-existing peripheral neuropathy to compare calcium/magnesium supplementation *vs* placebo failed to show any significant difference among treatment arms in the rate of moderate or severe neuropathy[114].

For all these reasons, whether the clinical outcome of an oxaliplatin-based first-line therapy is maintained in patients who had been already exposed to the drug in the adjuvant setting is unclear and few data are available on this regard. Recently, a retrospective study assessed the first-line RR to either FOLFIRI or FOLFOX in 32 patients with advanced CRC who had previously received adjuvant FOLFOX after radical surgery[115]. The median time between the beginning of adjuvant chemotherapy and disease recurrence was 1.7 years. The overall RR was 17% in the FOLFOX group *vs* 36% in the FOLFIRI group. Despite a trend in favor of FOLFIRI, the difference was not statistically significant ($P = 0.22$).

For patients with residual neurotoxicity at the time of disease recurrence, the stop-and-go strategy may be an appropriate option to avoid the side-effect worsening while still using an active agent. Two different randomized trials showed a clinically significant reduction in the rate of severe neurotoxicity with the use of this strategy[116,117]. In conclusion, an oxaliplatin-based regimen could still be an option for patients without or with minimal residual neurotoxicity that become metastatic

after at least 12 mo since the end of an oxaliplatin-based adjuvant therapy. Oppositely, for those who relapse early (within 12 mo) or still have clinically significant neurotoxicity, it is reasonable to choose a regimen without oxaliplatin and delay as much as possible the reintroduction of the neurotoxic drug.

CONCLUSION

The landscape of CRC treatment is changing very fast, and the availability of new therapeutic options has created new challenges and generated more complicated treatment algorithms. In conclusion, we would like to suggest the reader short possible answers to the initial questions. Undoubtedly, the optimal choice of the first-line treatment is still of great importance. When considering this choice, patients' performance status, comorbidities and desires should be considered as well as the ultimate goal of the treatment and the molecular features of the tumor. An highly intensive regimen is particularly indicated for younger patients without comorbid conditions or for those patients with aggressive colorectal carcinomas (symptomatic, bulky disease or BRAF mutant tumors). The application of a deeper molecular analysis not only helps identifying those patients who may benefit the most from EGFR-inhibitors but also has a prognostic value. In the majority of cases with RAS and BRAF wild-type status, a first-line combination with an EGFR-inhibitor seems to be the preferred treatment option, while the antiangiogenic strategy should be pursued in those with RAS mutated tumors or when a less aggressive treatment is favoured. The exposition to oxaliplatin in the adjuvant setting may somehow limit its use in the advanced phases of the disease due to possible cumulative neurotoxicity. Randomized trials, however, are verifying if a shorter oxaliplatin-based adjuvant treatment may be equally protecting and less toxic. Notably, many other new molecules are being studied in randomized trials and, hopefully, results of those studies will help clinicians further refining the current treatment paradigms.

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TOPIC HIGHLIGHT

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Neo-adjuvant radiotherapy in rectal cancer

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Abstract

In rectal cancer treatment, attention has focused on the local primary tumour and the regional tumour cell deposits to diminish the risk of a loco-regional recurrence. Several large randomized trials have also shown that combinations of surgery, radiotherapy and chemotherapy have markedly reduced the risk of a loco-regional recurrence, but this has not yet had any major influence on overall survival. The best results have been achieved when the radiotherapy has been given preoperatively. Preoperative radiotherapy improves loco-regional control even when surgery has been optimized to improve lateral clearance, *i.e.*, when a total mesorectal excision has been performed. The relative reduction is then 50%-70%. The value of radiotherapy has not been tested in combination with more extensive surgery including lateral lymph node clearance, as practised in some Asian countries. Many details about how the radiotherapy is performed are still open for discussion, and practice varies between countries. A highly fractionated radiation schedule (5 Gy × 5), proven efficacious in many trials, has gained much popularity in some countries, whereas a conventionally fractionated regimen (1.8-2.0 Gy × 25-28), often combined with chemotherapy, is used in other countries. The additional therapy adds morbidity to the morbidity that surgery causes, and should therefore be administered only when the risk of loco-regional recurrence is sufficiently high. The best integration of the weakest modality, to date the drugs (conventional cytotoxics and biologicals) is not known. A new generation of trials exploring the best sequence of treatments is required. Furthermore, there is a great need to develop predictors of response, so that treatment can be further individualized and not solely based upon clinical factors and anatomic imaging.

Key words: Chemotherapy; Chemoradiotherapy; Local control; Multidisciplinary; Organ preservation; Radiotherapy; Randomized trials; Rectal cancer

Core tip: Neo-adjuvant radiotherapy is beneficial to many rectal cancer patients since it reduces the risk of a local failure. Provided surgery is optimized, it does not substantially improve overall survival. This review describes the results of the randomized trials that form the basis for the present treatment recommendations. It also pinpoints reasons for differences in the care of rectal cancer patients seen worldwide. Finally, the concept of organ preservation is critically discussed.

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INTRODUCTION

Colorectal cancer is the third most common cancer worldwide and the second or third most common cause of cancer death. One third of the cancers arise in the rectum, the rest in the colon and most cases are adenocarcinomas. Survival has for decades been less favourable

in rectal than in colon cancer, but this is no longer the case[1-4]. Efforts to decrease rectal cancer loco-regional recurrence rates by better staging, improved surgery and incorporation of radiotherapy are the most likely reasons for the presently slightly better 5-year survival rates in rectal cancer. The local recurrence rates have also decreased from 30%-40% a few decades ago down to 5%-10% or even lower in some recent studies, and this has influenced survival in certain population-based studies. Survival still differs extensively between countries, and differences in therapy traditions are probably a major reason for this[5].

Radical removal of the primary rectal cancer, together with all regional tumour cell deposits are prerequisites for cure, although occasional local recurrences can be salvaged by (chemo)radiotherapy [(C)RT] and secondary surgery. Avoidance of persistent or recurrent tumour in the pelvis is important, even if cure cannot be achieved since uncontrolled pelvic growth is usually associated with severe symptoms. Even if overall survival is not improved, improved local control is a legitimate outcome of different interventions in rectal cancer. The primary tumour in the bowel is usually not the major problem unless it grows extensively towards organs not readily removed. In these patients, preoperative therapy with the aim of sterilizing macroscopic tumour cells in the periphery of the tumour is required. The most prevalent clinical problem is rather to eradicate the microscopic tumour cell deposits, adjacent to the primary which the surgeon does not always manage to remove with a standard surgical approach, today usually encompassing a total (or partial) mesorectal excision (TME). In Japan and other Asian countries, more extensive surgery with lateral node excision is performed in patients with high risk tumours[6], whereas in the western world, pre- or previously also postoperative (C)RT have been used to kill the subclinical tumour cells not removed by surgery. The (C)RT is then administered as adjuvant therapy after surgery, and as neo-adjuvant therapy before surgery.

An important aim is, thus, to treat so that the risk of residual disease in the pelvis is very low or preferably less than 5% in the population, in which curative treatment is intended. This should be possible in all but the few ($\leqslant$ 10%) cases, who present with a fixed tumour growing into a non-readily resectable organ. At the same time, as little acute and late morbidity as possible should be aimed at. Surgery, particularly if extensive, may give rise to substantial morbidity and the additional treatments, whether given pre- or post-operatively, increase both acute and late morbidity. Thus, all additional treatments, as well as more extensive surgery, should be given only when the expected gains are sufficiently large to motivate the increased morbidity.

This review about the value of radiotherapy to improve loco-regional control and overall survival in rectal cancer is based upon a systematic approach to the scientific literature. The available literature has been identified in several systematic overviews and meta-analyses[7-11]. It gives in addition some personal comments on observed developments during the past decades about sphincter- or organ preservation, where we lack good evidence of beneficial effects from controlled clinical trials.

Diagnosis and staging of rectal cancers

Appropriate diagnosis and staging are fundamental as regards choice of therapy. Tumours with distal extension to 15 cm or less from the anal margin (as measured by rigid sigmoidoscopy) are classified as rectal, and more proximal tumours as colonic. Others, *e.g.*, in Japan[12], prefer to separate colon and rectal cancers at the peritoneal reflection, or about 9-12 cm from the anal verge. Since the localization of the tumour in relation to other organs and structures and thus, the distance from the anal verge, is important for outcome and treatment, cancers between 10 and 15 cm are, in this author's opinion, best discussed as rectal cancers since radiotherapy (RT) is an important component of therapy, even if this is less common than for lower rectal cancers (0-10 cm)[13]. Lateral lymph node involvement is, however, rare in tumours above the peritoneal reflection[14].

Rectal MRI is recommended for staging in order to select preoperative treatment and extent of surgery, although endoscopic ultrasonography can be used for the earliest tumours[15,16]. If MRI and ultrasound are combined, a study claimed that accuracy was improved[17]. The TNM staging system should be used. At present, the latest version 7 from 2010 is preferred by most, even if it shows marked interobserver variations in defining stages Ⅱ and Ⅲ[18]. There is a need for further subclassification of clinical stage T3 (cT3) (Table 1) in order to individualize therapy, *i.e.*, to decide whether surgery alone is appropriate or whether preoperative RT alone or with chemotherapy (CRT) should be recommended.

Subdivision of rectal cancer with different therapeutic strategies

In order to define the extent of surgery and whether neo-adjuvant (or preoperative) (C)RT is required, rectal cancers can be divided into four groups, very early (some cT1), early (cT1-2, some cT3), intermediate (most cT3, some cT4) and locally advanced (some cT3, most cT4). Other factors than clinical T-stage, such as tumour height, closeness to the mesorectal fascia (mrf), potentially the circumferential margin (crm) (preoperatively, the term mrf is better than crm, since the crm cannot be defined until after surgery[19]), nodal (cN)-stage and vascular and nerve invasion are also relevant. It is not possible to precisely define which T and N sub-stages that belong to these groups. The terms "very favourable", "favourable or early or good", "intermediate or bad", and "locally advanced or ugly" can be used for categorizing the rectal cancers into these clinical subgroups. This subdivision (Table 2) is clinically relevant since primary treatment differs.

In many recent studies, the term "locally advanced" has been used for the "intermediate/bad" group, but is

Table 1 Tumor node metastasis-7 classification (2010) with subclassification of stage T3

TNM	Extension to
Tis	Carcinoma in situ: intraepithelial or invasion of lamina propria
T1	Submucosa
T2	Muscularis propria
T3	Subserosa/perirectal tissue
T3a[1]	Less than 1 mm
T3b	1-5 mm
T3c	5-15 mm
T3d	15+ mm
T4	Perforation into visceral peritoneum (a) or invasion to other organs (b)
N1	1-3 regional nodes involved
N1a	1 lymph node
N1b	2-3 lymph nodes
N1c	Small deposits in the fat
N2	4 or more regional nodes involved
N2a	4-6 lymph nodes
N2b	7 or more lymph nodes
M1	Distant metastases
M1a	1 distant organ or set of lymph nodes
M1b	More than 1 organ or to the peritoneum

[1]This subclassification is based upon an evaluation using magnetic resonance imaging prior to treatment decision is clinically valuable, and recommended in this review. It can be used also in the histopathological classification but is not validated and not incorporated in TNM version 7. TNM: Tumor node metastasis.

Table 2 Subgrouping of localized rectal cancer assessed by magnetic resonance imaging[1] and the recommended primary treatment

Favourable "good" group	Intermediate "bad" group	Advanced "ugly" group
Mid/upper rectum	Mid/upper rectum	
T1-3b	T3c/d	T3 mrf positive
Low rectum T1-2, T3a	low rectum also	T4 with overgrowth
N0	includes T3b	to prostate, seminal
mrf clear	T4 with peritoneal or	vesicles, base of urinary
	vaginal involvement	bladder, pelvic side walls
	only	or floor, sacrum positive
	N1/N2	lateral lymph nodes
	mrf clear	
5 yr LFR[2] < 10%	5 yr LFR[2] 10%-20%	5 yr LFR[2] 20%-100%
5 yr DFR[3] < 15%	5 yr DFR[3] 15%-60%	5 yr DFR 30%-80%
Primary surgery	Preop 5 × 5 Gy with	Preop CRT or 5 × 5 Gy
(TME)[4]	immediate surgery[5]	with delayed surgery[6]

[1]The algorithm (modified from[102] with permission from the publisher Informa) does not primarily address the risk of systemic disease, although this risk also increases with the presence of many of "the risk factors"; however, not necessarily parallel to the local failure rate (LFR). The algorithm is also "too simplified", in that a other factors like size of the mesorectum, anterior or posterior location, extramural vascular invasion (EMVI+) are also relevant. [2]Calculated in the group of patients planned for surgery, *i.e.*, irrespective of the surgical outcome. The table are valid if the surgeon is an experienced rectal cancer surgeon and no pre-treatment is given. [3]The 5-year risk of distant failure (DFR) is also given, although this risk is not well established. Risk factors detectable on magnetic resonance imaging for distant failure are N2 (versus N0 and N1), EMVI+, mrf+ and all T4 (a and b, see Table 1). These are also the risk factors used in the ongoing trial[88], where patients at high risk failing systemically are included. [4]A local procedure is possible in a few patients [chiefly pT1, sm1 (+ 2), N0]. This group is in the text referred to as "very favourable". [5]Preoperative chemotherapy (CRT) is also a valid option according to international clinical guidelines[21]. [6]CRT means chemoradiotherapy to 50.4 Gy in 1.8 Gy fractions with 5-fluorouracil (capecitabine). 5 × 5 Gy with delayed surgery should be used only in patients not fit for CRT.

best reserved for the truly "locally advanced/ugly" tumours[9,13,20]. Even if there is variability in what is called locally advanced there is consensus about the need to subgroup along these lines[13,21,22]. Subgrouping is an important step towards individualized therapy. Major discrepancies do, however, exist as regards which treatment is selected for these subgroups (Table 2).

Different treatment principles in the world

There is marked difference in how the subclinical tumour deposits often seen in tumours below the peritoneal reflection are managed in Asia and in the rest of the world. Surgical removal of the lateral nodes on one or both sides has been the preferred option in Asia[6,23], whereas the rest of the world has explored the value of radiation, in addition to surgery for the primary tumour in the bowel, to kill the tumour deposits. Since radiation does not selectively irradiate the lateral nodes, but also includes the primary tumour and the mesorectal nodes, the need for a meticulous surgical dissection technique has not been the same in the Western world as in Asia. Both extensive surgery and additional radiotherapy increase morbidity. It is not known which of the two alternatives is most efficient in eradicating all tumour cells, *i.e.*, preventing a local failure and which alternative results in the least morbidity since no randomized studies have compared the two strategies. Inter-trial comparisons have reported that the results are similar at specialized centres[24]. It is, however, probably more efficient to remove all subclinical cancer deposits using radiation rather than surgery, unless one can dissect in a surgical plane. The morbidity caused by extensive surgery is very different from that caused by external RT and less extensive surgery, although the relevance of this on patient well-being differs between cultures.

In the Western world, preoperative RT was mainly explored in Europe whereas postoperative RT was explored in the US. A few small studies showed that postoperative CRT was better than postoperative RT in preventing local recurrence and that combined treatment was more effective than surgery alone. A NIH Consensus Conference and a subsequent NCI report in the early 1990s stated that postoperative CRT should be standard treatment in rectal cancer stages Ⅱ and Ⅲ[25,26].

In Europe, several randomized trials compared surgery alone versus preoperative RT and surgery. These studies showed a relative reduction in local failure rates of 50%-60% if the radiation dose was moderately high (Table 3). If the radiation dose was lower, corresponding to a biologically effective dose (BED) below 30 Gy[7], no or a more limited effect was shown. As a consequence, preoperative RT was recommended as routine therapy in many European countries[13](Table 3).

Table 3 Major randomized radiotherapy trials in primary rectal cancer[1]

Study	Inclusion time	No of patients	Treatments			Radiation technique[2]	Increased postop death	Local recurrence			Increased survival	Comments
			Surgery alone	Preop (C)RT	Postop (C)RT			Surgery alone	Preop RT + surgery	Postop RT		
Pre-TME era												
MRC1[91]	1975-78	824	Yes	5 Gy × 1 2 Gy × 10		AP-PA	No	43%	45% 47%		No	Very low radiation dose, no benefit
EORTC[92]	1976-81	466	Yes	2,3 Gy × 15		AP-PA	No	28%	14%[1]		No	Decreased local recurrence risk
Bergen[93]	1976-85	169	Yes	1.75 Gy × 18		AP-PA	No	24%	17%		No	Marginally decreased local recurrence risk, comparably low dose
Stockholm Ⅰ[94]	1980-87	849	Yes	5 Gy × 5		AP-PA	Yes	28%	14%[2]	-	No	Increased postop death (8% *vs* 2%), large target, suboptimal technique, decreased local recurrence risk. Increased risk late complications
Uppsala[95]	1980-85	471	-	5.1 Gy × 5	2 Gy × 30	3D-C on RT	No	-	13%[1]	22%	No	Preop 5 Gy × 5 is better than postop RT (60 Gy). Increased risk of late complications after postop RT
S:t Marks[96]	1980-84	395	Yes	5 Gy × 3		AP-PA	Yes	24%	17%		No	Increased postop death (9% *vs* 4%)
MRC2[97]	1981-89	279	Yes	2 Gy × 20		AP-PA	No	46%	36%[1]		No	Slightly reduced risk of local failure, tendency to improved survival (HR = 0.79, 95%CI: 0.6-1.04)
North-West[98]	1982-86	284	Yes	5 Gy × 4		3D-C on RT	No	41%	18%[3]		No	Decreased local recurrence risk, 10 x 10 cm beams
SRCT[53,55]	1987-90	1110	Yes	5 Gy × 5		3D-C on RT	No	27%	12%[3]	-	Yes	Decreased local recurrence risk, no increased acute toxicity, some late toxicity after 10-15 yr
Stockholm Ⅱ[99]	1987-93	557	Yes	5 Gy × 5		3D-RT	Yes	25%	12%[3]	-	Yes	Overlaps to a large part SRCT, simplified radiation technique, tendency to increased postop mortality (4% *vs* 1%). Lower local recurrence risk, increased survival as in SRCT. Increased risk of late complications
Post-TME era												
EORTC 22921[38]	1993-03	1011	-	RT CRT[3]	RT CRT	3D-C on RT	No		17% 9%[2]		No	2 × 2 design, chemotherapy in addition to RT gives fewer local recurrences as first event than RT alone irrespective of whether concomitant (9%) or postoperative (10%), or both (8%), increased toxicity, no increased survival
FFCD 9203[37]	1993-03	742	-	RT CRT		3D-C on RT	No		17% 8%[1]		No	Preop CRT results in fewer local recurrences than preop RT, increased toxicity, no survival difference
AIO-94[30,100]	1995-02	823	-	CRT	CRT	3D-C on RT	No		6%[2]	13%	No	Preop CRT is less toxic and gives fewer local recurrences than postop CRT, no difference in survival
TME[54,101]	1996-99	1861	Yes	5 Gy × 5		3D-C on RT	No	11%	5%[3]		No	No increased postop mortality. Decreased local recurrence risk even with TME, no improved survival, some risk of increased late complications after 5-10 yr
LARCS[39]	1998-03	207	-	RT CRT		3D-C on RT	No		33% 18%[1]		Yes	The only study in "ugly" rectal cancers, preop CRT gives better local control and better disease and cancer specific survival, tendency towards better survival (66% *vs* 53% after 5 yr). Increased acute and possibly late toxicity from CRT
MRC-CR07[31]	1998-05	1350	-	5 Gy × 5	CRT if CRM+	3D-C on RT	No		5%[2]	11%	Yes	Preop 5 Gy × 5 better than postop CRT if CRM+, marginally increased survival. No increase in late complications (3-5 yr)

Polish[33]	1999-02	312	-	5 Gy × 5 CRT	3D-C on RT	No	11% 16%	No	First study that shows less risk of acute toxicity from 5 × 5 compared with preop CRT, no difference in local recurrence and survival or late complications (3-5 yr)
TROG[34]	2001-06	326	-	5 Gy × 5 CRT	3D-C on RT	No	7% 4%	No	Same design as the Polish study, same results

[1]Only large studies of relevance for present treatment recommendations are included. Patients with tumours considered to be resectable were included in all studies but one (LARCS). Staging has varied considerably over the years, but most included patients belonged to the intermediate group (bad) except in the LARCS study where most tumours were locally advanced (ugly). [2]AP-PA, anterior posterior beams with no blocking, meaning high radiation doses to large normal tissue volumes, 3D-CRT, 3D-conformed radiotherapy, 3 or 4 beams with blocking of normal tissues that did not contain tumour cells. 3D-RT (in the Stockholm Ⅱ study) means 4 beams but no blocking. [3]CRT means chemoradiotherapy with 1.8-2 Gy daily to 45-50.4 Gy. RT means the same radiotherapy as in the CRT arm without chemotherapy. [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$. TME: Total (or partial) mesorectal excision; CRT: Chemotherapy.

How is the radiotherapy best given?

For about two decades, four questions have been particularly discussed, *viz*. (1) should the RT be given before or after surgery; (2) should it be long-course or short-course; (3) should the long-course RT be given alone or with chemotherapy? In Europe researchers were not universally convinced of the advantages of adding concomitant chemotherapy considering the increased toxicity[27], as stated in the US documents. Furthermore; (4) could sphincter-saving surgery be increased after preoperative CRT? More recently, a fifth question; and (5) has attracted much interest, *viz*. if it is possible to avoid major surgery, *i.e.*, to preserve the organ, in patients who respond well to the preoperative CRT.

Pre- or postoperative radiotherapy?

A randomized trial showed at an early stage that preoperative short-course RT (5 fractions of 5 Gy in one week) was more effective and less toxic than postoperative long-course RT (Table 3). In the trial[28,29], significantly fewer local recurrences (13% *vs* 22%, $P < 0.05$) was seen in the group of patients randomized to the brief preoperative schedule than to an "optimized" postoperative schedule (high total radiation dose, 60 Gy in 7-8 wk, only given to high risk groups, stages Ⅱ + Ⅲ). Subsequently, several trials comparing preoperative CRT with postoperative CRT were initiated. The only completed trial[30] again showed that preoperative therapy was more efficient and less toxic than postoperative. In the trial, fewer local recurrences (6% *vs* 13%, $P < 0.01$) were seen in the group receiving preoperative CRT (Table 3). The preoperative treatment was also less toxic. No difference in survival was detected. Superiority of preoperative short-course RT over postoperative CRT was also shown in the MRC-CR07-trial; local recurrences were less commonly seen in the preoperatively irradiated group (5% *vs* 11%, $P < 0.01$)[31]. Most of the world has now accepted that additional (C)RT in rectal cancer should be given before, *i.e.*, neo-adjuvant, rather than after surgery. An analysis of data from the randomized studies also indicated that preoperative RT is more dose-efficient than postoperative RT[32].

Short- or long-course radiotherapy?

The question of whether the preoperative RT is best given as a short-course (5 Gy × 5) schedule or as long-course conventionally fractionated RT (1.8-2.0 Gy × 25-28) has been ongoing since the first results of the Uppsala trial were published in 1985[28], and the matter has not yet been settled. The potential advantages and disadvantages of the two fractionation schedules are presented in Table 4. The most recent RT trial in rectal cancer in Sweden, the Stockholm Ⅲ trial recently closed patient entry (Jan 2013). It has compared the different fractionation schedules in 845 patients randomized to either 5 Gy × 5 with immediate surgery, 5 Gy × 5 with delayed (4-8 wk) surgery and 2 Gy × 25, likewise with delayed surgery. Results of the primary outcome, local recurrences, will be available in 2015. Two other trials including 316 and 326 patients, respectively, could not find any differences in local recurrence rates, disease-free (DFS) and overall survival (OS) between the groups randomized to short-course RT alone or long-course CRT[33,34]. A German trial[35] with a similar design was initiated in 2004. No data has yet been released.

The short-course schedule has gained much popularity in Northern European countries where the health care system is rarely dependent upon private initiatives, whereas the long-course schedule is preferred in countries where physician and hospital budgets are influenced by the number of treatments given. Reimbursement has thus influenced routines, although this is seldom officially admitted. Many concerns have been expressed about the long-term consequences of hypofractionated RT. There is considerable evidence that the short-course schedule results in long-term morbidity, and the scale of that morbidity is well known[36]. The long-term morbidity of CRT, whether given pre- or

Table 4 Main differences between and potential advantages of short-course and long-course preoperative radiotherapy in intermediate (bad) rectal cancers[1]

	Short-course	Long-course
Total (physical) radiation dose	25 Gy	45-50.4 Gy
Fraction size/number of fractions	5 Gy/5	1.8-2 Gy/23-28
Radiation duration	1 wk	4.5-5.5 wk
BED[2], acute effects	37.5 Gy	37.5-44.4 Gy
BED[2], late effects	66.7	72-84 Gy
Overall treatment time	About 10 d	10-14 wk
Demands of radiation resources	Planning + 5 fractions	Planning + 23-28 fractions
Concomitant chemotherapy[3]	No	Yes
Acute toxicity	Minimal	More
Late toxicity	Present, considered limited in the "bad" group	Present, but not extensively studied. Anticipated to be higher than after short-course
Down-sizing/down-staging	No[4]	Yes[5]

[1]In locally advanced (ugly) tumours, long-course CRT is the preferred option although short-course RT with a delay to surgery is an option if CRT is not tolerated because of high age or co-morbidity; [2]Biologically effective dose according to the time-corrected linear quadratic model. Major uncertainties exist in the relative biological efficacy of the fractionation schedules concerning the acute, antitumour effects. The parameters selected for the acute effects were those used in the meta-analyses from 2001[7], even if they can be criticized and probably are incorrect. For late effects, an α/β of 3 Gy with no time correction is used. The anticipated antitumour effects do not thus differ substantially and late toxicity is at least not higher with short-course RT; [3]Improved local control with long-course RT, increased acute toxicity and probably also late toxicity. Should not be given with short-course RT; [4]Seen after short-course RT with delayed surgery; [5]Not relevant in these intermediate tumours (unless organ-preservation is aimed at), however, relevant in locally advanced (ugly) tumours. BED: Biologically effective dose.

postoperatively has not been studied systematically with the result that the extent of late morbidity is not precisely known. Both options, short-course 5 Gy × 5 and long-course CRT are considered valid in the intermediate group of rectal cancers, according to recent clinical guidelines[13,21]. The demands of radiation resources and the acute toxicity are much higher using long-course CRT than using short-course 5 Gy × 5. It is possible to conclude from the randomized trials that they have similar efficacy and do not differ in the risk of late toxicity; therefore, it is surprising to this author that they are considered equally valid (Table 4).

Radiotherapy alone or with chemotherapy?

Three randomized trials, two in the intermediate group[37,38] and one in the locally advanced, ugly group[39], have provided an answer to the third question. Local control was better in the combined treatment arm in all three studies, whereas a significant survival gain was only seen in the trial including locally advanced cancers[9,39]. Whenever a patient with a locally advanced, ugly rectal cancer receives preoperative treatment, CRT should be used unless the patient cannot tolerate this treatment. It should, however, be recognized that the gains from the chemotherapy addition are rather limited and come with a rather high price with significantly increased acute toxicity[11], and in all probability also increased late toxicity (see below).

The drug most extensively used to sensitize the RT has been 5-fluorouracil (5-FU), although oral capecitabine gives the same potentiation of the effects, and is more convenient[40]. Other oral fluoropyrimidines such as UFT[41,42] have also been explored, but have not yet been the subject of randomized trials. Combinations of 5-FU and other cytotoxic drugs such as oxaliplatin and irinotecan, and targeted drugs, have been extensively explored during the past decade. Multiple phase Ⅱ studies in so-called "locally advanced rectal cancer" have claimed superior results [more down-sizing, higher pathological complete (pCR) rates]. It is likely that these apparently favourable results depend upon the inclusion of mainly early or intermediate cancers. Five large randomized trials have failed to show any superior results from the addition of oxaliplatin[43-47]. When cetuximab was added to CRT with capecitabine and neo-adjuvant chemotherapy with capecitabine-oxaliplatin in a randomized phase Ⅱ study, the primary endpoint, pCR rate, was not increased, but more radiological responses (89% *vs* 72%, $P = 0.002$) and improved OS (96% *vs* 81% at 3 years, $P = 0.04$) were seen in the KRAS wild-type population ($n = 90$)[48]. These results need confirmation.

Sphincter preservation, organ preservation

Trials, again chiefly run in Europe, have explored whether long-course (C)RT with a delay before surgery could increase sphincter preservation rates, whereas others took it for granted that this was the case. The trials could not show that this occurred to any meaningful extent[49]. The hopes about improved chances of sphincter saving influenced routines in many countries, particularly in Southern Europe, Germany and the United States. At present, hopes about organ preservation (see below) influence treatment decisions at many centres.

TREATMENT ACCORDING TO RISK GROUP

Very favourable rectal cancer

In the earliest rectal cancers, chiefly the malignant polyps [Haggitt 1-3, T1 sm 1(-2?) N0], a local procedure, *e.g.*, using the transanal endoscopic microsurgery (TEM) technique, is sufficient for cure[50,51]. If the resection is not radical (R0), there are signs of vessel invasion, poor differentiation or if the tumour infiltrates more deeply into the submucosa (Haggit 4, T1) or is a T2 tumour, the risk of recurrence is too high (≥ 10%) and the patient should be recommended postoperative CRT or, more safely, major (TME) surgery. If the cancer diagnosis is biopsy-verified, presurgical CRT is preferred if the intent is to perform a local procedure[50]. As an alternative to local surgery, alone or with CRT, local RT (brachytherapy

or contact therapy using the Papillon technique) can be used. Experience of these treatments is limited outside specialized centres[52] and more prospective studies are required before they could be a part of clinical routines.

Favourable, "good" rectal cancers

In these cases cT1-2, some early cT3, N0 [cT3a(-b) and clear mrf (mrf-) according to MRI], "good" group, surgery alone using the TME technique is appropriate, since the risk of local failure is low unless the tumour is at the level of the levators[13]. Although the large randomized trials have indicated that short-course RT even further reduces local recurrence rates[31,53,54], surgery alone is recommended since the addition of preoperative RT results in overtreatment of too many individuals[13].

Intermediate, "bad" rectal cancers

In this group most cT3 [cT3(b)c+ without threatened or involved mrf (mrf-) according to MRI], some cT4 (*e.g.*, vaginal or peritoneal involvement only, N+), preoperative RT is recommended since the risk of local failure is not negligible (> 8%-10%), even if proper surgery is performed. Even in the absence of signs of extramural growth on ultrasound or MRI (cT2) in very low tumours (0-5 cm), preoperative RT may be indicated because the distance to the mrf or the levator muscles is very small. Surgery alone, often an abdomino-perineal excision, will then again result in unacceptably high local recurrence rates. Twenty-five Gy delivered during one week and followed by immediate surgery (< 10 d from the first radiation fraction) has in randomized trials reduced the risk of local failure by 50%-70% *vs* surgery alone[31,53-55]. The relative efficacy is likely to be the same irrespective of tumour height, although this was not seen in the TME trial[54]. CRT to 46-50.4 Gy, 1.8-2.0 Gy/fraction with 5-FU (bolus, continuous infusion or peroral) is an alternative, although it is more demanding and not proven to be more effective[33,34,37,38]. CRT is preferred in low rectal cancers even at centres that otherwise use 5 Gy × 5. It must be stressed that RT (or CRT) cannot compensate for poor surgery. Surgery should aim at clear resection margins (crm-); therefore, in low rectal cancers requiring an abdomino-perineal excision, it is important to do the dissection so that a "waist" is avoided. As described above, two European trials[37,38] showed that the addition of 5-FU improved local control with a reduced risk of local failure as first event. After 5 years these were 17% in the preoperative RT arms alone and 8%-9% in the CRT arms. In the EORTC trial, the same reduction was seen whether the chemotherapy was administered concomitantly with the RT, only postoperatively or both pre- and postoperatively. Two randomized trials (Polish, TROG 1.04) could not detect any statistically significant differences in local recurrence rates, DFS and OS after preoperative 5 × 5 Gy or preoperative CRT (5-FU + 50.4 Gy)[33,34]. In the TROG study, numerically more recurrences were seen in the group randomized to 5 Gy × 5 (6/48 *vs* 1/31, P = 0.21)[34]. In the MRC-CR07-trial including 1350 patients, preoperative 5 × 5 Gy was randomly compared with postoperative CRT if the crm was positive. Local recurrence rates favoured the preoperative arm (5% *vs* 17%, $P < 0.001$)[31]. DFS was also superior in the preoperative arm (HR = 0.76, $P = 0.01$) whereas OS did not differ significantly (HR = 0.91, $P = 0.04$).

Locally advanced, "ugly" rectal cancers

In the locally advanced, frequently non-resectable cases [cT3 mrf+, cT4 with overgrowth to other organs (cT4b)], preoperative CRT, 50.4 Gy, 1.8 Gy/fraction with concomitant 5-FU-based therapy should be used[9,13,39], followed by radical surgery 6-8 wk later. In a Nordic randomized trial (cT4NXM0), local control was significantly better after 5 years in the CRT arm (5-FU + 50 Gy) than in the RT only arm (82% *vs* 67%, $P = 0.03$). Also DFS and cancer-specific survival were significantly better in the combined modality arm, whereas OS did not differ significantly (66% *vs* 53%, $P = 0.09$)[39].

In very old patients (≥ 80-85 years) and in patients not fit for CRT, 5 × 5 Gy with a delay of approximately 8 wk before surgery is an alternative option, based upon three retrospectively analyzed patient series revealing favourable results[56-58]. A randomized trial will in all probability never be performed in this patient group, which is not considered to tolerate standard therapy.

Organ preservation?

Apart from the earliest tumours that can be treated with a local procedure or local RT, as described above, it has become increasingly popular to give CRT, then wait and restage the tumour[59-62]. If no signs of remaining tumour/no viable tumour cells are found when biopsies are performed, major surgery is not performed and the patient is monitored closely for at least 5 years. The hypothesis is that potential lymph node metastases have been eradicated parallel with the response of the primary tumour. Although this occurs in some patients, this strategy has not been the subject of properly controlled prospective studies. This excellent response will not be frequent in the intermediate and locally advanced cases[63,64], but only in early cases. The cell kill effect of available CRT schedules is too small.

No major surgery and no rectal excision in very low tumours can be clearly beneficial for individuals who run a high risk of surgical therapy or who cannot accept a stoma. However, the disadvantages for many patients are seldom discussed. In most patients with an early rectal cancer, a low anterior resection alone is the reference treatment. Cure rates are high and morbidity is only a result of the surgery. If these patients are treated with the aim of organ preservation, all will receive CRT with its acute morbidity. Patients who respond with a clinical complete remission (cCR), and are not operated are the ones potentially having a benefit of a wait-and-see approach, although they will all suffer from the long-term toxicity that can be seen after CRT. If the tumour is located in the lower rectum, at least part of the sphincters

must be included in the irradiated volume, and suboptimal anal function can be a result. Those who do not achieve a cCR or those who recur during follow-up will require major surgery. These patients will thus suffer the morbidity from both CRT and major surgery. It is presently not possible to know the proportion of patients who do not require major surgery. With the CRT schedules available today, the group of patients having a true advantage is most probably much smaller than the group of patients who suffer extra morbidity.

Radiation therapy volumes and doses

In the "intermediate/bad" group, with the aim of lowering the risk of local failure, the primary tumour with the mesorectum and lymph nodes outside the mesorectum, at risk to contain tumour cells more than exceptionally should be irradiated[65,66]. In the "early/good" group before or after a local procedure, only mesorectal nodes are at sufficient risk to be involved. The appropriate dose to subclinical disease should with 5-FU chemotherapy be at least 45 Gy in 1.8-2.0 Gy fractions. The relative reduction in local failure rates is then in the order of 50%-60%, and subsequently there is room for improvement. A boost of about 4-6 Gy in 2-4 fractions to the primary tumour is sometimes given[67]. A brachytherapy boost has also been tried; however, without any apparent advantage[68]. The clinical problem is not the primary tumour in the bowel, unless you aim at organ preservation (see above).

In the "locally advanced/ugly" tumours, the target is basically the same as in the intermediate group, although the primary tumour extends more laterally and more lymph nodes can be at risk. In these patients, a lateral boost to areas where it can be difficult to surgically remove all cells can be indicated[69]. It is not primarily motivated to boost the centre of the tumour, *e.g.*, where the PET-uptake is the highest, if this can surgically be removed.

The entire mesorectum is in most cases at great risk of having tumour deposits and should be included in the clinical target volume (CTV). In high tumours it is sufficient to include the 4 cm distal to the tumour. Besides the mesorectal nodes, the presacral nodes up to the level of S1-2 should be included in CTV. If presacral nodes are radiologically involved, the upper border of CTV should be even higher. Local recurrences above S1-2 are infrequent[70-72]. The lateral nodes, including the internal iliac nodes up to the bifurcation of the common iliac arteries should be included in tumours below the peritoneal reflection, *i.e.*, in tumours up to about 9-12 cm from the anal verge[73]. The risk of lateral node involvement in the Western world is not precisely known, but studies from Asia show that these lymph nodes are rarely involved in low-mid rectal pT1-2 tumours and in high tumours irrespective of T-stage[14,74]. External iliac nodes should only be included if an anterior organ such as the urinary bladder, prostate or female sexual organs are involved. The medial inguinal nodes need only to be prophylactically included when the tumour grows below the dentate line[75].

The ischiorectal fossae should be included only when the levator muscles and the internal and external sphincters are involved. The fascia inside the levators is considered to be a strong barrier to tumour cell penetration[76]. Other opinions have been expressed[65].

Late toxicity from rectal cancer radiotherapy

The prevention of a local failure with the severe morbidity this may have must be weighed against the morbidity from (C)RT that all treated patients can develop. From the Swedish and Dutch randomized trials, the morbidity after 5 × 5 Gy RT is well described and reviewed in[36]. Increased risks of poor anal and sexual function, small bowel toxicity with obstruction and secondary malignancies have been reported. Studies have tried to estimate what minimal absolute gain should be present for patients to prefer RT. These studies are difficult to interpret, although many patients accept an absolute 3% difference in local recurrence risk for the known morbidity risks of RT[77].

After having treated rectal cancer patients for over 30 years, and thus, seeing many patients with a local recurrence during the first part of the period, and being actively involved in research aimed at estimating the extent of late toxicity up to 20 years after the RT, it is my opinion that an absolute risk reduction of approximately 5% motivates the recommendation to irradiate. The recommendations given above, as well as in recent consensus statements[13,21] reflect this opinion. Furthermore, and very importantly, the RT we give today, and the RT that routinely can be given in only a few years[66,78,79], will mean even less late toxicity than that seen in the follow-up studies of the RT deli vered during the 1980s-1990s. Better understanding of internal movements will also allow more precise delivery of the radiation dose[80] and of dose-response relationships for *e.g.*, faecal incontinence[81].

An important question is the late toxicity from 5 × 5 Gy compared with the late toxicity seen after 46-50 Gy in 25-28 fractions, usually administered with 5-FU. The long-term morbidity from 5 Gy × 5 up to at least 10 years follow-up (with yesterday's techniques) is known from studies including thousands of patients. This knowledge is not as solid from CRT. The Polish[33] and the MRC-CR07 trials[31] could not detect any differences between 5 × 5 Gy and CRT to 46-50 Gy after 4 years of follow-up. The short-course schedule uses a high fraction size of 5 Gy, compared with 1.8-2.0 Gy, whereas the total dose is less (25 Gy compared to 46-50 Gy). Both the fraction size and the total dose are relevant. The relationship between total dose, fraction size and late toxicity is, however, complex.

Another question is whether the addition of 5-FU increases late toxicity. In one of the two larger randomized trials in the intermediate risk group[37,38], the addition of 5-FU negatively affected global QoL, social functioning and diarrhoea. Almost 60% of the patients suffered from faecal incontinence, impairing their social life[82]. In the trial in locally advanced/ugly cancers, more patients

had a stoma or a poor anal function in the CRT group than in the RT group (89% *vs* 70%, $P = 0.046$)[83]. If this means that the addition of chemotherapy results in more late toxicity or if this difference reflects the survival of patients with more advanced tumours in the CRT group cannot be deduced. No differences in QoL were seen after 4-8 years[84].

CONCLUSION

During the past three decades, a severely disabling condition for many rectal cancer patients, viz a local failure with uncontrolled growth of the cancer in the perineum and pelvis has disappeared, although, unfortunately, not yet at all centres. Multiple trials have confirmed the superiority of what can presently be considered as recommended care and treatment (Table 2). A multidisciplinary approach has been a must in this development, at present formalized as (weekly) multidisciplinary team (MDT) meetings, during which all patients are discussed before the first treatment decision, postoperatively, and at critical time points during the course of the disease. Many countries have successfully launched quality assurance and quality control programmes in rectal cancer surgery[85,86]. It is important that, besides surgical details, RT and CRT details are also fully integrated in the programmes.

Practically all details in the care of the patients have been the subject of prospective, frequently randomized trials. It should, however, also be recognized that many uncertainties about what is the best treatment still exist. Furthermore, alternative approaches to attain low local failure rates and improved survival together with as little negative consequences from the disease and its treatment as possible, also exist.

The trials have repeatedly shown that RT, whether alone or with chemotherapy, should be given before surgery to have the best efficacy and least toxicity. This was shown as early as 1985, but is only recently unanimously agreed upon. It is also a belief that systemic treatment, being the weakest part of the therapy, should be given before and not after the surgery in order to have greatest efficacy. Progression of the local primary should then not occur during the systemic treatment, presently requiring a duration of 5-6 mo. The discovery that the short-course schedule results in substantial down-staging, is tolerable and permits full chemotherapy starting soon after the RT[56,87], has led to the next generation of studies, such as the multicentre "RAPIDO" trial[88]. Patients with ugly rectal cancers at high risk to recur are randomized to the present standard, CRT, surgery and adjuvant chemotherapy (even if not all consider this standard[89]) and an experimental arm with 5 × 5 Gy, neo-adjuvant chemotherapy and surgery at the end. A Polish study, likewise in locally advanced, unresectable rectal cancer, with a similar design is also ongoing[90]. In an interim analysis after 97 randomized patients, no major differences in acute toxicity and local efficacy were seen between the control group receiving CRT (50.4 Gy with 5-FU/FA/oxaliplatin) and the experimental group (5 × 5 Gy followed by 3 FOLFOX-4 cycles preoperatively). No postoperative therapy is scheduled.

During the past 30 years, a better understanding of the molecular mechanisms involved in tumour development and progression has placed great expectations on improved diagnosis, staging, prognostic evaluation and selection of the individually best therapy. Much new and valuable information has been created, but no new clinically valuable markers have been identified. The number of mm's from the most peripheral part of the rectal tumour to the mrf (or crm postoperatively) is most informative. No predictor of which pre- (or post-)operative treatment to choose is available. The efforts to translate basic knowledge into clinically useful information must be intensified or explored along other paths. Sampling of representative and sufficient tumour material for diagnosis and research prior to, during and after therapy may help. Functional imaging showing where to sample, may be helpful. We need predictors and must find better ways of identifying them than has been possible in the past.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Colorectal cancer: Current imaging methods and future perspectives for the diagnosis, staging and therapeutic response evaluation

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Abstract

In the last 10 years the mortality rate of colorectal cancer (CRC) has decreased by more than 20% due to the rising developments in diagnostic techniques and optimization of surgical, neoadjuvant and palliative therapies. Diagnostic methods currently used in the evaluation of CRC are heterogeneous and can vary within the countries and the institutions. This article aims to discuss in depth currently applied imaging modalities such as virtual computed tomography colonoscopy, endorectal ultrasound, computed tomography (CT) and magnetic resonance imaging (MRI) in the diagnosis of CRC. Special focus is put on the potential of recent diagnostic developments as diffusion weighted imaging MRI, MRI biomarkers (dynamic enhanced MRI), positron emission tomography with 2-(fluorine-18)-fluoro-2-deoxy-D-glucose (FDG-PET) combined with computed tomography (PET/CT) and new hepatobiliary MRI contrast agents. The precise role, advantage and disadvantages of these modalities are evaluated controversially in local staging, metastatic spread and treatment monitoring of CRC. Finally, the authors will touch upon the future perspectives in functional imaging evaluating the role of integrated FDG-PET/CT with perfusion CT, MRI spectroscopy of primary CRC and hepatic transit time analysis using contrast enhanced ultrasound and MRI in the detection of liver metastases. Validation of these newer imaging techniques may lead to significant improvements in the management of patients with colorectal cancer.

Key words: Colorectal cancer; Imaging; Staging; Computed tomography; Magnetic resonance imaging; Diffusion weighted imaging; Contrast enhanced ultrasound

Core tip: This state-of-the-art review article covers current and future contribution of various imaging modalities in the diagnosis of colorectal cancer. Primary local staging, metastatic spread, restaging and posttreatment response evaluation are discussed in depth using emerging techniques such as virtual computed tomography (CT) colonoscopy, endorectal ultrasound and positron emission tomography/CT. The role and indications of more recently developed techniques as magnetic resonance imaging (MRI) with diffusion weighted images and hepatobiliary contrast materials are evaluated. The challenges and evolving role of functional imaging with MRI spectroscopy and hepatic transit time analysis using MRI and contrast enhanced ultrasound in the detection of liver metastases are also covered.

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INTRODUCTION

Colorectal cancer (CRC) is the second most common cause of cancer death in the western world, with a high lifetime incidence of 6%. The prognosis of CRC is like other tumors staging dependent and the 5 years survival lies in the range of 40%-60%. Due to optimization of surgical techniques, introduction of neoadjuvant therapies and recent developments in diagnostic imaging modalities, the mortality rate has decreased significantly by 20% in the last years.

Utilization of different imaging modalities in diagnosing of CRC vary between countries and institutions. While computed tomography virtual colonoscopy (CTC) is a validated tool in the primary diagnosis of CRC in the United States[1], this method is used with caution in many European countries due to radiation exposure and is thus not included as a screening modality in asymptomatic patients[2]. The pros and cons of this rapidly evolving diagnostic modality compared to endoscopy are discussed controversially.

Imaging for surgical planning depicts the relationship of the tumor to surgical key landmarks and shows the presence of metastatic disease. Imaging features enable preoperative evaluation of prognostic features, which may guide patient selection for specific (*e.g.*, neoadjuvant) therapy[3]. Recent developments in imaging technologies and validation of newer imaging techniques may lead to significant improvements in the management of patients with CRC. Diagnostic techniques such as diffusion weighted imaging (DWI), Fluorodeoxyglucose positron emission tomography (FDG-PET) and dynamic contrast enhanced magnetic resonance imaging (DCE-MRI) are increasingly used and have shown to be clinically useful in tumor characterization[4-6]. Newly developed techniques such as perfusion computed tomography (CT) and MRI spectroscopy allowing insights in tumor biology have shown promising results, however they are not yet validated for clinical practice[7,8].

This review discusses the current and future contribution of various imaging modalities to already established and recently developed techniques to improve the diagnosis for both-tumor detection and tumor characterization of CRC. In addition, the evolving role of newly developed methods for functional evaluation of otherwise "occult" hepatic metastases such as Doppler perfusion index (DPI)[9] and hepatic transit time (HTT) analysis using contrast enhanced MRI[10] will also be covered.

PRIMARY DIAGNOSIS OF COLORECTAL CANCER

Considering the high diagnostic performance, optical colonoscopy (OC) remains the gold-standard investigation in the early detection of CRC. Colonoscopy allows biopsy samples to be taken for definitive diagnosis with a simultaneous opportunity for a therapeutic polypectomy, therefore improving a long-term prevention of CRC deaths[11]. However, patients with tumor related stenosis, older patients and those with comorbidities are more likely to have an incomplete or difficult OC[12,13].

VIRTUAL CT COLONOSCOPY

In recent years the role of CTC as a potential alternative to endoscopy has been widely studied[14-16]. CTC image formation is based on the X-ray attenuation of low-density; high-intrinsic-contrast objects such as the air contained in the colonic lumen versus the large bowel walls, acting as an interface between intra luminal air and the extra luminal compartment. Low X-ray energy is sufficient to achieve diagnostic CTC images, resulting in a low radiation dose. If CTC is aimed at the sole examination of the colon (*e.g.*, for CRC screening purposes), the use of low radiation dose CT acquisition protocols is warranted. Conversely, regular dose CT protocols can be used if CTC is part of a CT examination in which all abdominal organs have to be investigated. This method is applied in patients with known CRC and incomplete OC, in whom CT plays a role for both complete assessment of the colonic lumen and for oncological staging (Figure 1). For adequate colonic distention to be achieved, air or carbon dioxide is usually delivered into the patients colon with a thin rectal catheter prior to CTC. Air has the advantage of no cost and the ease of administration, but is less tolerated as it is not absorbed by the colonic mucosa. Conversely, carbon dioxide is more comfortable as it is gradually absorbed by the colonic walls, although larger volumes must be supplied compared with air. In practical terms, administration of 1.0-1.5 liter of air or 3-4 liter of carbon dioxide is usually sufficient[17]. The CT acquisition is usually performed twice: in supine and prone position (or vice versa). This is to optimize the distention of the various colonic segments depending on gravitational compression by the surrounding abdominal structures, as well as to distinguish polyps which may be fixed to the bowel walls from fluid and/or fecal residues. Colonic distention is also favored by parenteral administration of spasmolytic agents, such as glucagon or hyoscine-N-butyl bromide, which inhibit peristalsis and reduce the tone of the parietal musculature. By orally administering positive contrast material (barium or iodine), fecal and fluid tagging can be performed, helping to distinguish fecal/fluid residues from parietal polyps. Tagged residual fluid can then be electronically removed from CTC images by means of a dedicated software. 3D reconstructions enable accurate quantification of polyp volume, which can be helpful in a follow-up to assess growth of the polyps. Research is in progress on subtracting solid tagged stool in patients who do not undergo cathartic cleansing.

Pickhardt *et al*[14] found CTC comparable to colonoscopy in detection of bigger colorectal polyps. Two meta-analysis studies showed a high sensitivity (100%) of CTC in the detection of colon cancer and 87.9% for adenomas less than 10 mm[18,19]. Despite such promising data, there

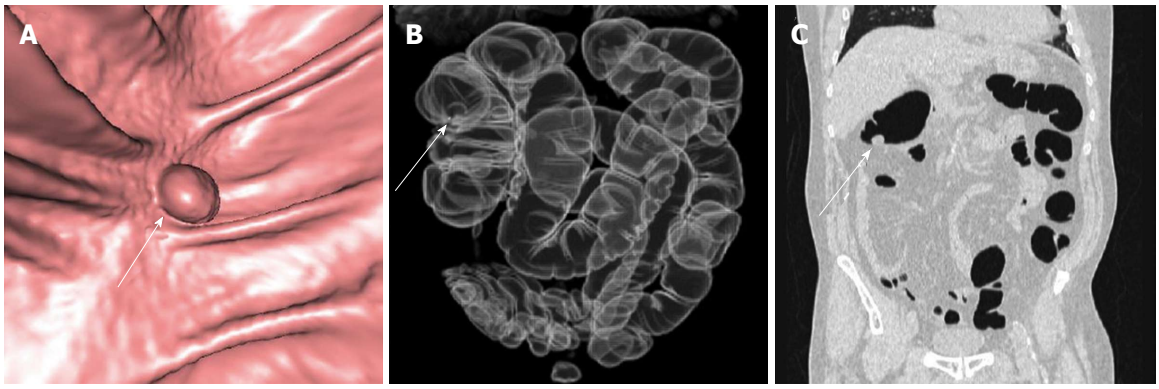

Figure 1 A 64-year-old male patient who underwent routine screening colonoscopy terminated due to severe discomfort. A: Virtual computed tomography (CT) colonoscopy detected a 1 cm polyp (arrow) in right colonic flexure, biopsy proved as adenocarcinoma; Fly-through with a 3D view of the polyp; B: The virtual X-ray reconstruction; C: Coronal reconstruction using the lung window shows the tumor clearly.

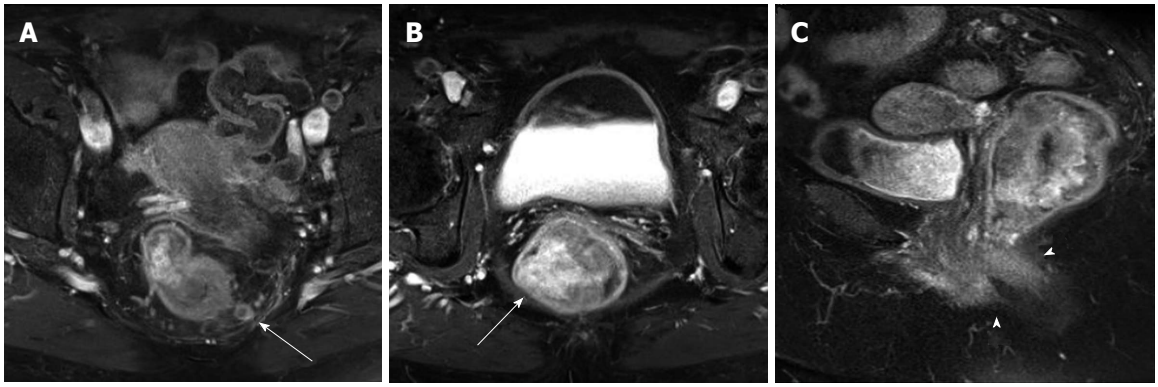

Figure 2 Abdominal magnetic resonance imaging for local staging of rectal adenocarcinoma in a 58-year-old female. A, B: Post-contrast fat-suppressed axial images show 7 cm long contrast enhancing neoplastic mass with lymph node metastases within the mesorectal fascia (arrow); C: Peripheral desmoplastic reaction (arrowheads) on T1 sagittal images.

is currently no transcontinental consensus on whether CTC should be used as a screening method in asymptomatic patients. Since 2008 CTC is recommended as a validated diagnostic tool by the American Cancer Society and is included among the screening tests of CRC[1]. This recommendation was revalidated in a recent large patient sample (1610 patients) multicenter randomized trial by Atkin *et al*[16], concluding that CTC is a similarly sensitive, less invasive alternative to colonoscopy. However, in many European countries the use of CTC as a screening method in asymptomatic populations is prohibited due to radiation related consequences and only advised in cases of incomplete preoperative colonoscopy[2].

An alternative method to CTC could be MRI colonoscopy which is not radiation exposure related[20]. However, currently there are insufficient study results available to recommend this method as a screening modality.

LOCAL STAGING OF CRC: MRI AND ENDORECTAL ULTRASOUND

The tumor node metastasis classification of the American Joint Committee on Cancer is the internationally accepted standard for the staging of CRC[21]. The accurate diagnosis of local tumour extension, location, T stage, potential circumferential resection margins, mesorectal fascial involvement and extramural or venous invasion is essential for defining the treatment strategy. For this reason, MRI is the recommended modality for initial staging, due to its high accuracy for the definition of localization, determining the total extension and the relationship of the tumor to the peritoneal reflection[22]. Furthermore, MRI is accurate in measuring the distance between the anorectal junction and the distal part of the tumor. It is also accurate for determining the length of the tumor. Although it has been the standard in the past, it is inappropriate to use the term circumferential resection margins (CRM) for initial clinical staging before surgery, since CRM can be defined only postoperatively by the surgical plane. The tumor growth on primary staging MRI should be best described in relation to an anatomical structure, like the mesorectal fascia[23]. Most staging failures with MRI occur in the differentiation of T2 stage and borderline T3 stage with overstaging as the main cause of errors[24]. Overstaging is often caused by desmoplastic reactions[5] and it is difficult to distinguish on MRI between spiculation in the perirectal fat caused by fibrosis alone (stage pT2) and spiculation caused by fibrosis that contains tumor cells in stage pT3 (Figure 2).

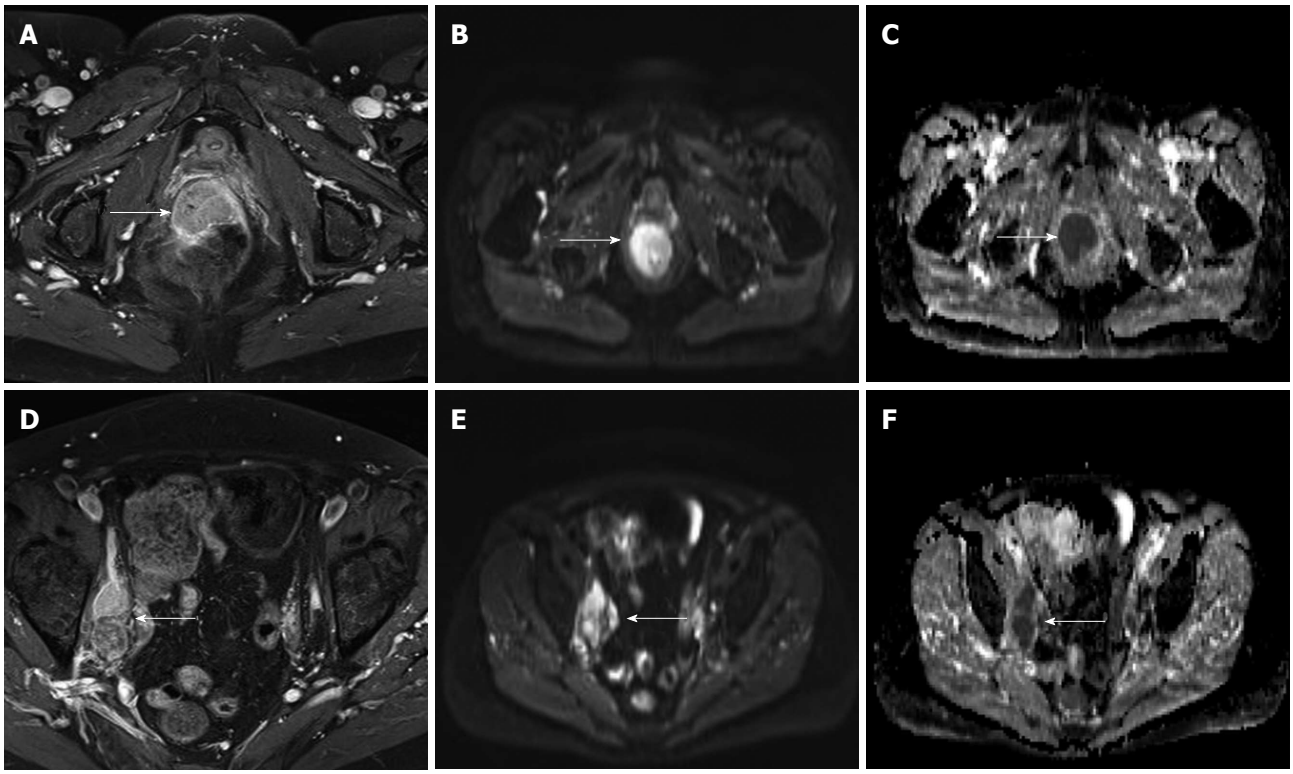

Figure 3 A 58-year-old female with biopsy-proven adenocarcinoma of the rectum. A: Post-contrast fat-suppressed axial T1 images show a contrast-enhancing mass (arrow), extending from rectum into the anal canal and invading the posterior aspect of the vagina; B, E: Both, the primary tumor and the lymph node metastases, show an hyperintense signal on diffusion weighted imaging; C, F: An reduced apparent diffusion coefficient reflecting the tight tumor cellularity; D: Enlarged, contrast-enhancing lymph nodes along the right iliac axis (arrow).

Although previous studies have not shown much advantage of dedicated phased-array coils[25], our clinical experience is positive and at our institution we use phased-array coils as a standard in the primary diagnosis of colorectal cancer. The advantage of high spatial resolution with a large field of view is making phased-array MRI suitable for staging of both superficial and advanced rectal tumors. A standard phased-array MRI protocol for rectal cancer consists of T2-weighted turbo spin-echo (TSE) MR sequences with high spatial resolution. The strength of T2-weighted turbo spin-echo MRI of rectal cancer is that fat tissue remains high in signal intensity. In this way, the tumor contrasts well with the surrounding fat tissue, and even very thin hypointense structures such as the mesorectal fascia can always be identified independent of the body habitus of the patient, owing to the high contrast between the hypointense fascia and the hyperintense fat tissue in and outside the mesorectum[5].

At our institution, phased-array MRI for primary rectal cancer staging is performed at 1.5 Tesla (Siemens Avanto and Espree, all Siemens Healtcare, Erlangen, Germany) and 3.0 Tesla (Siemens Prisma, Skyra and Verio). The protocol consists of a T2 SPACE 1.0 mm isovoxel sequence, a standard echo planar imaging sequence for diffusion (b-values: 0, 40, 400 and 800 s/mm^2) including an apparent diffusion coefficient (ADC) map and a T1 TSE Dixon sequence with fat saturation (FS) and calculation of in-/opposed-/fat- and water maps before contrast administration (Figure 3). Post contrast sequences are just a standard transversal T1 TSE FS (SL 5 mm) and a T1 VIBE FS 1.2 mm isovoxel. The pre- and post contrast isovoxel sequences can be reconstructed in line with and perpendicular to the individual tumor.

Endorectal Ultrasound (ERUS) is now an established modality for evaluation of the integrity of the rectal wall layers. With accuracies for T staging varying between 69% and 97%, endorectal ultrasonography (US) is currently the most accurate imaging modality for the assessment of T1 tumors[2]. ERUS and endorectal MRI have similar accuracy in the differentiation between superficial (T1 and T2) and T3 tumors[26]. However, endorectal MRI is related to high costs, limited availability and is less patient friendly. Consequently, endorectal MRI is not recommended by the European Society for Medical Oncology Guidelines as a preferred imaging modality for clinical T stage in colorectal cancer[22].

METASTATIC SPREADING OF CRC

In 25% of patients with colonic cancer and in 18% of patients with rectal cancer, metastases are present at the time of the first diagnosis. The most frequently used imaging modalities for the detection of CRC metastases are US, CT, MRI and PET/CT[27]. Current National Comprehensive Cancer Network guidelines for initial staging of CRC suggest the use of chest/abdomen/pelvis CT or

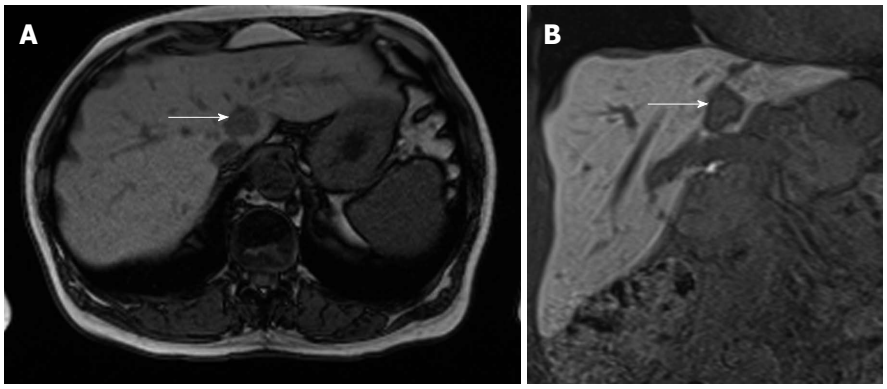

Figure 4 A 63-year-old female with colorectal cancer and suspected liver metastasis. A: Primovist images acquired 10 min *p.i.*, during the hepatobiliary phase using a T1 VIBE isovoxel sequence with coronal orientation; B: Due to the high resolution axial reconstructions are also done routinely. The lesion in segment I (arrow) is clearly demarcated as a contrast defect because of the missing hepatocytes in the metastasis while the other parts of the liver show a bright contrast enhancement.

MRI, while FDG-PET/CT is reserved for surveillance or problem solving.

N staging

ERUS, CT and MRI use the size as the main criterion in the assessment of nodal involvement, although the lymph node size is not an ideal indicator of metastasis and lacks sufficient accuracy for clinical decision-making[28]. FDG-PET gives better insight in tumor biology, however, due to limited spatial resolution it does not allow for reliable detection of small lymph node metastases. FDG-PET/CT may provide additional information and could increase the accuracy of lymph node involvement significantly with a sensitivity and specificity of 51% and 85% for local lymph nodes and 62% and 92%, for distant lymph nodes[29].

M staging

Correct detection of hepatic and pulmonary metastases can be challenging considering the possible difficulties in differentiation with benign lesions in these organs. CT has a better diagnostic performance (sensitivity 74%-84%, specificity 95%-96%) compared to US in detection of CRC liver metastases[30]. A meta analysis of prospective studies comparing FDG-PET, MRI, and CT demonstrated a superior performance of MRI over the other two modalities on a lesion-by-lesion basis of the liver and in particular in evaluating lesions less than 1 cm in size (sensitivity 80%-88% and specificity 93%-97%)[6].

Recently, DWI and hepatobiliary phase MRI with new hepatobiliary contrast agents have been integrated for the detection of liver metastases demonstrating improved sensitivity over routine MRI alone[31]. The newest hepatobiliary contrast agent available is Gd-EOB Primovist® in Europe and Eovist® in United States and Canada (Bayer Healthcare, Leverkusen, Germany). Uptake of contrast within the hepatocytes results in peak parenchymal enhancement approximately 10-20 min *p.i.*, referred to as the hepatobiliary phase. As expected, lesions like metastases without containing hepatocytes are strongly hypointense compared to the surrounding enhanced parenchyma in this phase (Figure 4).

For the detection of pulmonary metastases imaging can be limited to chest X-ray. Although CT detects more lesions compared to chest X-ray (CXR), a large number of these lesions (4%-42%) does not allow for a definitive diagnosis. Only one quarter of unspecified pulmonary lesions found on CT are demonstrated to be metastases, therefore the high sensitivity of CT cannot guarantee important benefit for the patients[32]. This concept is supported by a recent study showing that preoperative staging chest CT is not beneficial for CRC patients without liver and lymph node metastasis on abdominal and pelvic CT who had a negative initial CXR finding[33].

RESTAGING: THERAPEUTIC RESPONSE EVALUATION

General considerations

Patients after primary tumor resection and those treated with chemoradiation therapy (CRT) for locally advanced CRC require a regular post treatment evaluation. Within the first 5 years after curative therapy there is an increased chance for a locoregional relapse (3%-24%), occurrence of distant metastases (25%) and for developing metachronous secondary tumors (1.5%-10%). The introduction of preoperative adjuvant CRT has led to a reduction in local recurrency rates and has become standard of care for patients with locally advanced rectal cancer.

Several studies investigating the role of imaging for restaging after CRT suggest that neither MRI nor ERUS or FDG-PET are sufficiently accurate for identifying the true complete responders with positive predictive values ranging from 17%-50%[34-36]. T2 weighted MRI has been standardly used for local restaging (Figure 5). Many recent reports have shown that DWI MRI may be useful for the response evaluation after CRT[37,38]. DWI has shown to be feasible as an early marker of treatment response because cell death and vascular alterations typically occur before size changes. It also has been proved that DWI in addition to standard MRI significantly improves the performance of radiologists to select complete therapy responders compared to standard MRI only[39,40]. In a recent systematic review and meta analysis study including 1556 patients from thirty-three studies MRI has shown to be useful for tumor-free CRM restaging, however nodal staging remained challenging[41]. High b-value DWI is sensitive for detecting the location of lymph nodes, but

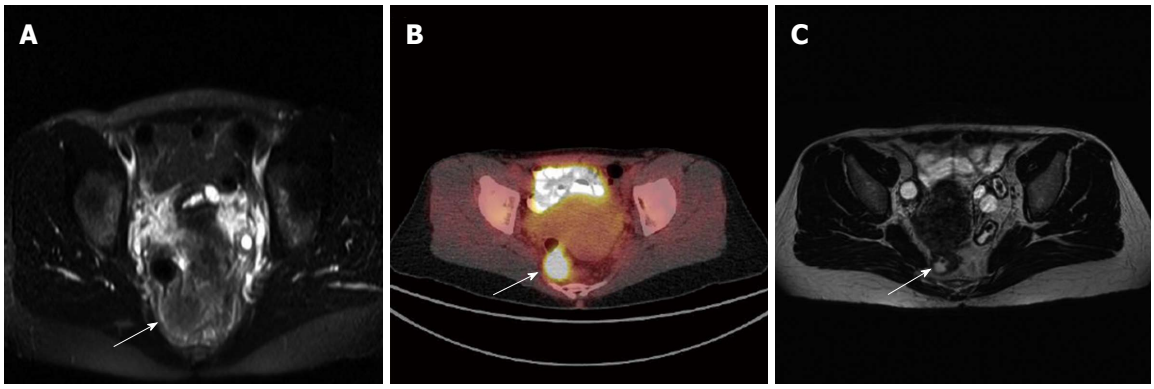

Figure 5 Initial rectal cancer staging of a 48 year old female. A: Occlusion of the rectum by solid tumor on magnetic resonance imaging (MRI) (arrow); B: Corresponding high Fluorodeoxyglucose metabolism in the Fluorodeoxyglucose positron emission tomography/computed tomography; C: Post treatment (chemoradiation therapy) magnetic resonance imaging shows a clear tumor size reduction (arrow) with a continuing lumen after chemoradiation therapy.

characterization of neoplastic nodes yields false-negative results and reactive hyperplastic nodes false-positive results.

It has been reported that transient decrease in the ADC may occur early in treatment related to cellular swelling, reduction in the blood flow or extravascular-extracellular space[42]. However, early decreases in ADC values are not consistently seen and it has recently been reported that increases in ADC value with therapy response occur within 3-7 d in responding CRC patients treated with chemotherapy[43]. Therefore the utilization of ADC values in the CRC evaluation needs further standardization and validation.

The role of FDG-PET in evaluating of recurrent colon cancer is controversial. Some of the previously published studies showed high specificity of this modality (up to 98%) based on evident FDG reduction after adjuvant CRT[44]. Metabolic changes in response to treatment occur before any structurally detectable change (*e.g.*, tumor shrinkage). In the neoadjuvant setting, serial FDG-PET examinations may aid treatment planning to decide the appropriate length of neoadjuvant chemotherapy to maximize tumor response before surgical resection. In this setting, FDG-PET could lead to changes in therapies for those patients with tumors that show no metabolic change[45]. On the contrary, other studies suggest that when radiation therapy is applied, FDG-PET cannot reliably identify pathologic complete response to CRT due to radiation related increased FDG uptake by rectal mucosa resulting in high false-positive data[46,47]. For this reason, when using FDG-PET to monitor tumor response, it is not advocated within the first 4 wk after completion of CRT. FDG-PET/CT is a unique combination of the cross-sectional anatomic information provided by CT and the quantitative metabolic information provided by FDG-PET. In the past years, FDG-PET/CT has taken an important place in treatment response assessment[48]. Limitations of FDG-PET/CT are that the technique is cost- and time-consuming (utilizing about 1.5 h per patient) and is not widely available.

Considering a very limited benefit of CRC follow-up in stage I tumors, described as only 1% increase in patient survival[49], a regular follow-up in these patient group is not indicated. In patients with advanced primary CRC (stage Ⅱ and Ⅲ), US is advised for the follow-up of liver metastases. US has a slightly lower sensitivity compared to CT in the detection of liver metastases, however the performed studies did not show a convincing advantage of CT over US in evaluation of asymptomatic patients[50]. Therefore, abdominal US can be indicated as a cost-effective, widely available and relatively simple diagnostic modality in the follow-up of CRC liver metastases.

Up to 7% of all curatively treated patients with CRC develop distant pulmonary metastases which in 3.4%-30.0% are detected with chest X-Ray[51]. Therefore CRX evaluation can be sufficient in follow-up of asymptomatic patients.

For anatomic objective response evaluation criteria based on assessment of the size of the tumor or metastases, Response Evaluation Criteria in Solid Tumors (RECIST) have been developed[52]. RECIST uses unidimensional measurements of the sum of the longest lesion diameters of target lesions. At our institution we use a commercially available software for RECIST analysis (mint Lesion®, Mint Medical GmbH, Heidelberg, Gemany), which will be discussed below.

Software based follow-up

Proper response assessment and reporting of metastatic lesions are crucial. A major pitfall in tumor response monitoring is the increasing incidence of mixed response to chemotheraphy and subjective measurements of the lesions, *e.g.*, liver and lung metastases, also lesion measurements are time-consuming and can be investigator dependent. Computerized tools able to optimize the radiologist's workflow of the image reading process are spreading as the need for a systematic, standardized follow-up procedure grows. For example, the syngo® CT Oncology software (Siemens Healtcare, Erlangen, Germany) is able to perform automated measurement of neoplastic lesions helping to solve the long-standing issue of interobserver variability.

Another automated tool is mint Lesion® (Mint Medical GmbH, Heidelberg, Gemany), developed at the German Cancer Research Center (Heidelberg, Germany)

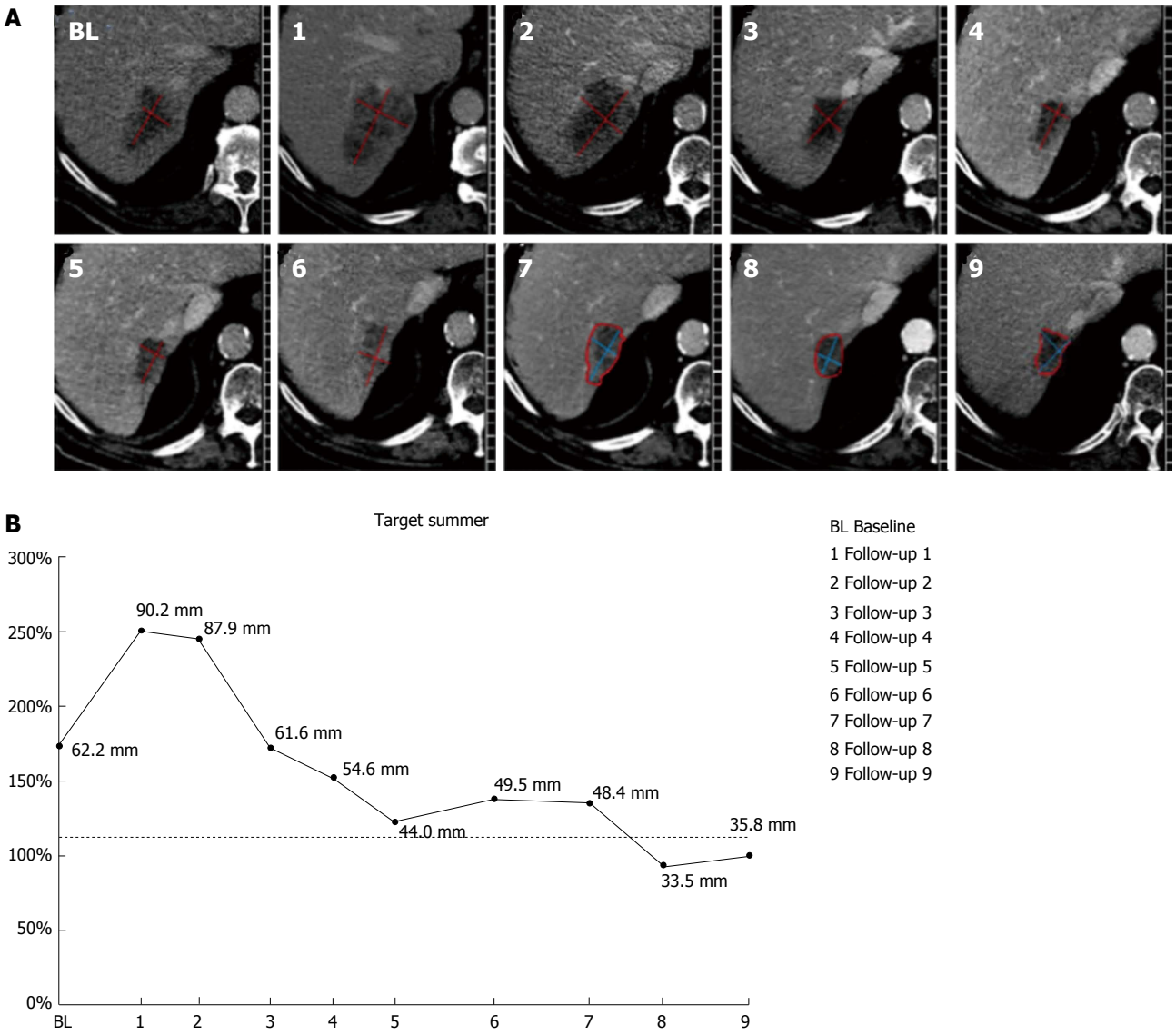

Figure 6 An automated software (mint Lesion®, Mint Medical GmbH, Heidelberg, Gemany) used at our Institution for tumor treatment response evaluation. A: Computed tomography images show liver metastasis in a patient with colorectal cancer on nine follow-up examinations; B: Graphical evaluation depicts the measurement of the lesion standardized throughout the whole staging period consisting of baseline (BL) and nine follow up assessments (1-9). Tumor response is evaluated according to Response Evaluation Criteria in Solid Tumors.

which is currently routinely used at our institution for oncological assessment. Connected with our Picture Archiving Computer System (PACS) the mint Lesion software is able to continually synchronize and upgrade its worklist retrieving and matching precedent patients' related studies allowing a workflow optimization. It covers management of patient cohorts in terms of disease and treatment, assessment of lesions with respect to the overall patient treatment course, statistical response evaluation in line with response criteria, and consistent and comprehensive automated reporting.

In the initial baseline assessment target and non-target lesions are defined. In subsequent follow-up exams the software is able to correlate and match images of the previous studies allowing a faster recognition of previously described lesions; by showing exactly how quantitative measurements (*i.e.*, volumetry, density and intensity) were performed in previous studies, interobserver variability is thus reduced. Apart from the reproducible measurements, assessment notes, treatment outcome statistics for patient cohorts and individual patients, mint Lesion® provides an automatically generated, consolidated visual and textual overview of a single treatment course (Figure 6). Graphical charts help to identify the dimensions of tumor load change with respect to baseline, nadir and previous exams. Therapy course overview is clearly depicted in the results and can be sent as digital imaging and communications in medicine to the PACS as well as actively included in the report. By such means, the standardization of the read workflow contributes to the assessment quality of longitudinal follow-up sequences providing comprehensible information for an interdisciplinary assessment of the therapy response by a tumor board.

Beyond resolution: Functional imaging

Functional imaging now has a growing role in colorec-

tal cancer assessment. Recent developments in imaging technologies and validation of these newer imaging techniques may lead to significant improvements in the management of patients with colorectal cancer.

To date, FDG-PET does not have an established role in primary diagnosis of colon cancer reflecting limited availability of resources and lack of convincing cost-benefit data[53]. This technique has low sensitivity revealing mucinous adenocarcinomas in which metabolic activity is low. Partial volume averaging and necrotic lesions may cause false-negative results, and incidental physiologic bowel FDG uptake or inflammation will produce increased tracer uptake, giving rise to false-positive findings that can mimic a tumor. The controversial role of FDG-PET in the posttreatment setting has been already discussed above. Prediction of the nodal status by CRC remains problematic. A novel nanoparticle MRI lymphographic agent - ultrasmall superparamagnetic iron oxide particles showed an overall sensitivity and specificity of 88% and 96% in the detection of lymph node metastases of CRC[54]. Regretfully these MRI contrast agents are not yet available for clinical practice.

Dynamic contrast-enhanced (DCE) CT and MRI have been described as potential prognostic biomarkers in CRC. The results of the studies evaluating DCE-CT as a biomarker for chemoradiation are controversial: while baseline low perfusion values were described to be associated with a poorer response in the study by Bellomi *et al*[55], another group reported the contrary[56]. DCE-MRI data uses two compartments for contrast agent accumulation: blood plasma and extravascular-extracellular space. K^{trans} (volume transfer constant between the blood plasma and the extravascular-extracellular space, the washout rate, measured in minutes^{-1}) and K_{ep} (rate constant between the extravascular-extracellular space back to the blood plasma, the washout rate, measured in minutes^{-1}) determine the transport between these two compartments. Rectal tumors with higher K^{trans} values at presentation appear to respond better to CRT than those with lower values. After CRT, usually K^{trans} values are reduced, while persistent raised values indicate residual active disease[57].

Experimental techniques in primary colorectal cancer diagnosis

In a study by Ng *et al*[58], CT texture features of primary colorectal cancer were studied in relation to 5-year overall survival rate. The authors studied the tumor heterogeneity using a range of parameters, including entropy, uniformity, kurtosis, skewness, and standard deviation of the pixel distribution histogram. According to this study tumors demonstrating less heterogeneity at fine filter levels were associated with poorer survival, concluding that the addition of texture analysis to staging contrast-enhanced CT may improve prognostication in patients with primary colorectal cancer. Goh *et al*[8] assessed an interobserver agreement in a prospective study with integrated FDG-PET/CT and perfusion CT to evaluate the relationship between tumor glucose metabolism and vascularization. FDG-PET/CT was used to localize the colorectal tumor, and CT coordinates were used to plan the subsequent perfusion. The study showed good intra- and interobserver agreement for the metabolic-flow differences, suggesting this approach as a robust parameter for clinical practice.

The role of MR Spectroscopy (MRS) has been of great interest in the recent years to improve the primary diagnosis of various cancer groups. In a small sample ex vivo prospective study on 24 subjects with colorectal cancer without neoadjuvant treatment, MRS was able to discriminate healthy from neoplastic tissue and to distinguish patients with different prognoses[7].

Functional imaging in liver metastases of colorectal cancer

The liver is the first organ most likely to develop distant metastases from CRC. Knowledge of hepatic metastatic involvement during identification of the primary tumor is therefore crucial. The idea is not new and we can follow several attempts to get access to that information back to the nineteen-eighties. The approach is to detect the arterialization of the liver blood supply during the onset and development of liver metastases. In a normal healthy individual approximately two thirds of the blood supply of the liver arrives *via* the portal vein and one third *via* the hepatic artery. During the development of liver metastases, this relation changes: the above mentioned arterialization occurs, which means the arterial portion of the liver blood supply increases while the portal vein portion decreases[59]. This has been shown first with technetium colloid scintigraphy to estimate the so called hepatic perfusion index (HPI) in overt liver metastases[60-62]. Meanwhile it has been shown that the hemodynamic changes occur already at an early microscopic stage of metastasis formation[63,64].

Leen *et al*[65] developed a Doppler ultrasound method to get a parameter similar to the HPI, the DPI, which gives the hepatic arterial blood flow relative to the portal venous flow. This ratio was raised in patients with liver metastases. The method demonstrated not only the possibility to detect overt liver metastases but also the arterialization due to occult metastases for the standard morphology based imaging methods. This study showed that patients with colorectal cancer, without liver metastases on first imaging and a raised DPI, had a much higher risk of developing liver metastases in the following five years than those with normal DPI. The DPI method thus seems to detect the presence of metastases which were occult to all other imaging modalities[9]. Unfortunately, DPI measurements are strongly operator dependent and other groups could not reproduce Leen's results[66,67]. HTT analysis of a microbubble ultrasound contrast agent has then been proposed as an alternative technique for detecting hepatic arterialization[68]. It was initially used to show arterialization in patients with hepatic cirrhosis[69,70]. Meanwhile several studies have shown that the method is able to detect hemodynamic changes in liver metastases but depends on the used contrast agent[10,71-73] (Table 1).

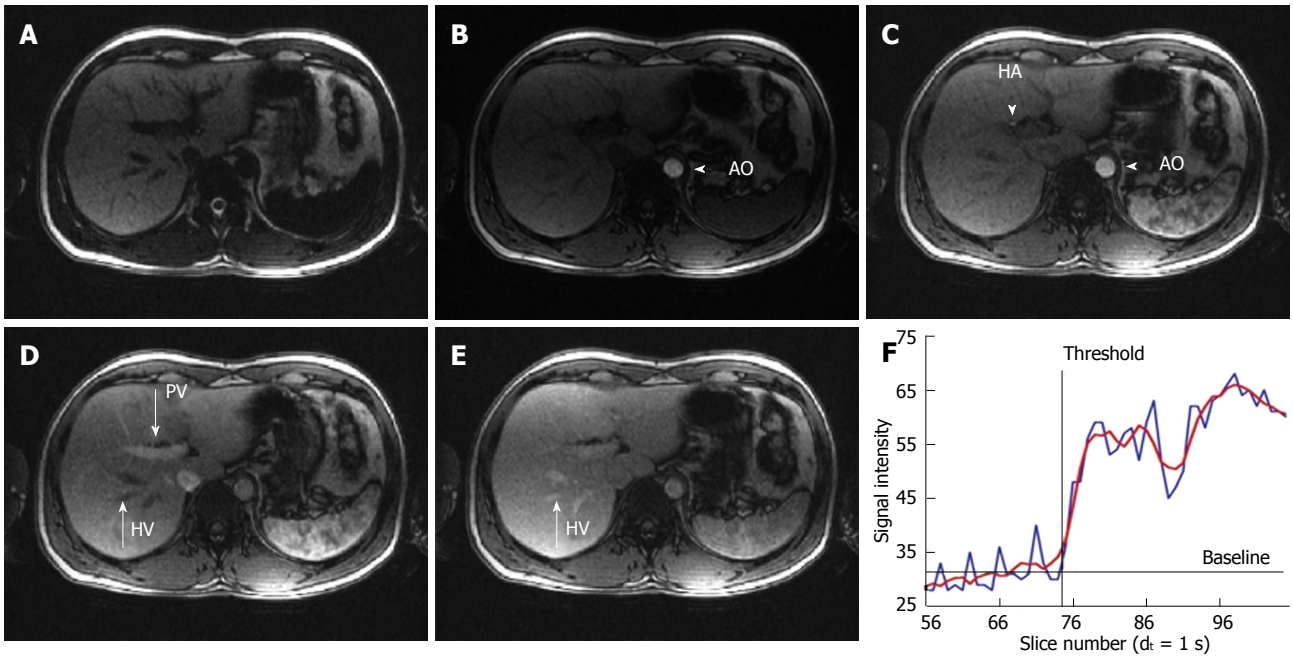

Figure 7 Magnetic resonance imaging images of the T1-weighted sequence for hepatic transit time analysis at different time points. A: Baseline image without contrast; B: Arterial phase with opacification of the aorta (AO); C: Arterial phase with opacification of the AO and the hepatic artery (HA); D: Portal venous phase with additional enhancement of the portal vein (PV); Note that the hepatic veins (HV) are still not enhanced; E: Venous phase with complete opacification of all vessels including the HV; F: Example of a typical time intensity curve acquired from a ROI placed at the position of the HA; The raw data in the graph (blue line) has a modulation due to patient breathing. Therefore the curve has to be fitted and smoothed (red line). The calculated baseline as well as threshold point, demonstrating the arrival time of the contrast agent is drawn.

Table 1 Summary of studies with measurement of hepatic transit times in subjects with and without the evidence of liver metastases

Study	Number of patients	Hepatic transit time (arterial to venous) (s)		contrast agent	modality
		Liver metastases	No liver metastases		
Bernatik *et al*[73]	28/36	6.7	15.4	Optison[1]	CEUS
Hohmann *et al*[10]	22/22	7.4	11.1	SonoVue[2]	CEUS
Zhang *et al*[71]	5/3	6.2	11.3	SonoVue[2]	CEUS
Haendl *et al*[72]	20/15	6.3	9.3	SonoVue[2]	CEUS
Haendl *et al*[72]	12/14	9.9	14.8	Levovist[3]	CEUS
Haendl *et al*[72]	20/15	6.3	9.2	Luminity[4]	CEUS
Hohmann *et al*[78]	20/21	7.1	13.5	MultiHance[2]	MRI

[1]Amersham, Little Chalfont, United Kingdom; [2]Bracco, Milano, Italy; [3]Bayer, Berlin, Germany; [4]Lantheus, N. Billerica, MA, United States. These are mainly studies using CEUS but also one study which used MRI. All other MRI studies used slightly different approaches and did not measure directly comparable values. CEUS: Contrast enhanced ultrasound; MRI: Magnetic resonance imaging.

There have also been other attempts to measure hepatic blood supply changes with CT and MRI. With CT this is usually a perfusion measurement with the calculation of different perfusion parameters such as hepatic artery and portal vein perfusion and the HPI[74,75]. The major drawback of CT measurements is radiation exposure and, therefore, most of the studies are animal studies. Even though the results are promising, there are probably no realistic possibilities for CT perfusion measurements in humans.

With MRI the approaches are different which are summarized under the term diffusion/perfusion measurements (Figure 7). Especially with focal or global perfusion methods, MRI seems to have great potential to detect hemodynamic changes due to focal liver lesions[76]. While the first studies just measured perfusion parameters in one single plane[77,78], this changed to measurements of the whole liver with 3D Datasets but with limited time resolution[79,80]. It should currently be possible to increase this time resolution in further studies. All the previous mentioned methods required intravenous contrast material, which might have an influence on the results similar to the results on MRI as it was shown with CEUS. Therefore new methods without contrast material, like hemodynamic response imaging, which has proven to show therapy response in experimental settings, are very promising[81].

Overall, for functional imaging in patients with colorectal cancer, MRI of the liver offers the widest variety of possibilities in the future. This might be essential for the detection of occult liver metastases at the time of first diagnosis of colorectal cancer and will then result in different therapeutic approaches due to the results of the measurement.

CONCLUSION

In recent years several attempts have been made to improve the diagnostic performance of imaging modalities for better characterization of CRC. To date, OC remains the most precise modality in the detection of primary CRC simultaneously allowing biopsy and therapeutic

polypectomy. Virtual CT colonoscopy is gaining importance as a potential alternative to OC, recently showing a similar diagnostic performance[16]. However, radiation exposure and the lack of instantaneous therapeutic possibilities remain a primary concern. To date, there are insufficient study results to recommend MR Colonoscopy as a screening modality.

MRI and ERUS at present show the best results in the local staging of rectal carcinoma[5,22,23]. MRI is the superior imaging modality for the evaluation of primary tumor location, extension and mesorectal fascia involvement. Overstaging remains problematic on MRI, related to difficulties in differentiating desmoplastic reaction caused by fibrosis alone (stage pT2) and by fibrosis that contains tumor cells (stage pT3). ERUS, with an accuracy of up to 97%, is currently the most accurate imaging modality in the assessment of T1 rectal tumor[2]. For the detection of CRC distant metastases, US and CT are the most advocated modalities. Although FDG-PET/CT shows an increased accuracy in metastatic lymph node assessment, utilization of this modality is limited and cannot be applied broadly. Recent studies support the concept that in preoperative staging chest CT is not beneficial and imaging of the patients without hepatic and lymphatic metastases can be limited to CXR[81].

Newer techniques in functional imaging may lead to significant improvements in the management of CRC. The hepatobiliary MRI contrast agent (Gd-EOB Primovist/Eovist®, Bayer Healthcare) is available to improve the detection of liver metastases and could be problem-solving in difficult cases. In treatment response monitoring, DWI is gaining a promising role as a reliable marker to improve MRI performance, however, characterization of metastatic lymph nodes remains challenging. Other MRI biomarkers in the treatment response evaluation such as Dynamic contrast enhanced MRI and perfusion CT might improve the insights in tumor biology to better characterize residual tumor. Experimental studies on MRI spectroscopy of primary CRC, MRI diffusion/perfusion and hepatic transit time analysis using MRI in the detection of metastatic liver disease are promising. However, further research in larger series is needed to be applicable in clinical practice.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Lymph node staging in colorectal cancer: Old controversies and recent advances

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Abstract

Outcome prediction based on tumor stage reflected by the American Joint Committee on Cancer (AJCC)/Union for International Cancer Control (UICC) tumor node metastasis (TNM) system is currently regarded as the strongest prognostic parameter for patients with colorectal cancer. For affected patients, the indication for adjuvant therapy is mainly guided by the presence of regional lymph node metastasis. In addition to the extent of surgical lymph node removal and the thoroughness of the pathologist in dissecting the resection specimen, several parameters that are related to the pathological work-up of the dissected nodes may affect the clinical significance of lymph node staging. These include changing definitions of lymph nodes, involved lymph nodes, and tumor deposits in different editions of the AJCC/UICC TNM system as well as the minimum number of nodes to be dissected. Methods to increase the lymph node yield in the fatty tissue include methylene blue injection and acetone compression. Outcome prediction based on the lymph node ratio, defined as the number of positive lymph nodes divided by the total number of retrieved nodes, may be superior to the absolute numbers of involved nodes. Extracapsular invasion has been identified as additional prognostic factor. Adding step sectioning and immunohistochemistry to the pathological work-up may result in higher accuracy of histological diagnosis. The clinical value of more recent technical advances, such as sentinel lymph node biopsy and molecular analysis of lymph nodes tissue still remains to be defined.

Key words: Colon cancer; Rectum cancer; Tumor staging; Lymph node metastasis; Prognosis; Sentinel lymph node; Lymph node ratio; Extracapsular invasion; Immunohistochemistry; Molecular analysis

Core tip: For patients with colorectal cancer, the indication for adjuvant therapy is mainly guided by the presence of regional lymph node metastasis. This review provides an in depth analysis of parameters affecting the clinical significance of lymph node staging, focusing on changing definitions of lymph nodes, involved lymph nodes, and tumor deposits in different editions of the American Joint Committee on Cancer/Union for International Cancer Control tumor node metastasis staging system, the minimum number of lymph nodes that should be evaluated, lymph node ratio, extracapsular invasion, sentinel node biopsy, and the potential benefit of ancillary techniques, such as immunohistochemistry and molecular analysis.

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INTRODUCTION

Colorectal cancer is one of the most common cancers worldwide. In the United States, approximately 102480

new cases of colon cancer and 40340 new cases of rectal cancer have been estimated for 2013. For the same time period, 50830 deaths from colorectal cancer have been calculated, accounting for about 9% of all cancer deaths[1].

Surgical resection is the treatment of choice for patients with locally confined disease. Outcome prediction based on tumor stage reflected by the American Joint Committee on Cancer (AJCC)/Union for International Cancer Control (UICC) tumor node metastasis (TNM) system is currently regarded as the strongest prognostic parameter. Adjuvant chemotherapy, which is primarily based on 5-fluorouracil, has decreased tumor recurrence in AJCC/UICC stage Ⅲ patients, while neoadjuvant chemotherapy and total mesorectal excision have improved local control in patients with rectal cancer. The indication for adjuvant therapy is mainly guided by the presence of regional lymph node metastasis[2-4].

A plethora of controversies exists how the evaluation of resected lymph nodes should be performed, many of these affecting the clinical significance of lymph node staging in daily routine practice (Table 1). This already starts with varying definitions of lymph nodes as such, lymph nodes involved by metastatic tumor tissue, and their differentiation from tumor deposits, as is reflected by changing criteria in different editions of the AJCC/UICC TNM staging system[5]. The number of examined lymph nodes has been identified as an additional important issue. Some investigators claim the lymph node ratio, defined as the number of positive lymph nodes divided by the total number of retrieved nodes, to be more important than the absolute number of positive nodes[6-9]. Likewise, the identification of extracapsular invasion by cancer cells may help to improve the prognostic significance of lymph node staging[10-13].

Manual dissection with subsequent histological assessment based on routinely hematoxylin and eosin (HE) stained slides is the standard approach in the examination of regional lymph nodes in cancer specimens (Figure 1)[14]. However, some studies have raised the suspicion that analysis based solely on HE stained slides is insufficient for a proper evaluation. This notion has led to the introduction of new techniques, such as sentinel node biopsy, immunohistochemical and molecular analyses in the work-up of cancer specimens[15].

In this review, we will refer to the controversies mentioned above in detail, focusing on both clinical impact and technical issues. Data for this review were compiled using MEDLINE/PubMed and Thomson Reuters Web of Science®, assessing articles published before August 2013. The search terms included colorectal cancer, colon cancer, rectum cancer, TNM classification, lymph node metastasis, lymph node ratio, extracapsular invasion, sentinel lymph node, immunohistochemistry, and molecular analysis. Only articles published in English were considered.

LYMPH NODE STAGING ACCORDING TO THE AJCC/UICC TNM SYSTEM

Quantitative lymph node evaluation has repeatedly been validated as a powerful prognostic tool in patients with colorectal cancer. In particular, the absolute number of positive nodes has been identified as a highly effective predictor of adverse outcome, as shown by worsening of prognosis with increasing number of lymph nodes involved by cancer[16,17].

Table 1 Parameters affecting the clinical significance of lymph node staging in colorectal cancer

Extent of surgical lymph node removal
Thoroughness of the pathologist in dissecting the resection specimen
Technical methods to increase lymph node yield
Methylene blue injection
Fat clearing
Acetone compression
Changing definitions of lymph nodes, involved lymph nodes, and tumor deposits in different editions of the AJCC/UICC TNM staging system
History of neoadjuvant treatment
Absolute number of retrieved lymph nodes
Absolute number of positive lymph nodes
Lymph node ratio
Presence of extracapsular invasion
Sentinel node biopsy
Number of histological sections
Use of immunohistochemistry to identify micrometastasis and/or isolated tumor cells
Use of molecular techniques to identify minimal tumor disease in lymph node tissue

AJCC: American Joint Committee on Cancer; UICC: Union for International Cancer Control; TNM: Tumor node metastasis.

Hence, in the AJCC/UICC staging system the prognostic stratification of nodal disease is based on the absolute number of positive lymph nodes. Difficulties, however, arise with respect to changing definitions of lymph nodes as such, involved lymph nodes, and/or tumor deposits (satellites) in different editions[5]. Tumor deposits are macroscopic or microscopic nests or nodules of cancer found in the pericolic and/or perirectal adipose tissue's lymph drainage area of a primary carcinoma (away from the leading edge of the infiltrating tumor) without histological evidence of residual lymph node in the nodule. They are histologically heterogeneous and may be seen associated with distinct anatomic structures, such as veins[18]. These deposits may represent discontinuous primary tumor spread, venous invasion with extravascular spread, or a totally replaced lymph node (Figure 2A-C).

The main differences between the different editions of the AJCC/UICC TNM system regarding lymph node staging are as follows: The 5th edition of the TNM system (TNM-5) introduced the 3 mm rule for their classification, providing a tool based exclusively on the size of the lesions[19]. The 6th edition (TNM-6) discarded the size criterion and referred to the contour of the lesions[20]. The 7th edition (TNM-7) focused on the differentiation of lymph node metastases from tumor deposits, including the latter in the pN category (pN_{1c})[21]. Details are presented in Table 2.

Nagtegaal *et al*[5] have proven lymph node staging according to TNM-5 to be superior to TNM-6, as demon-

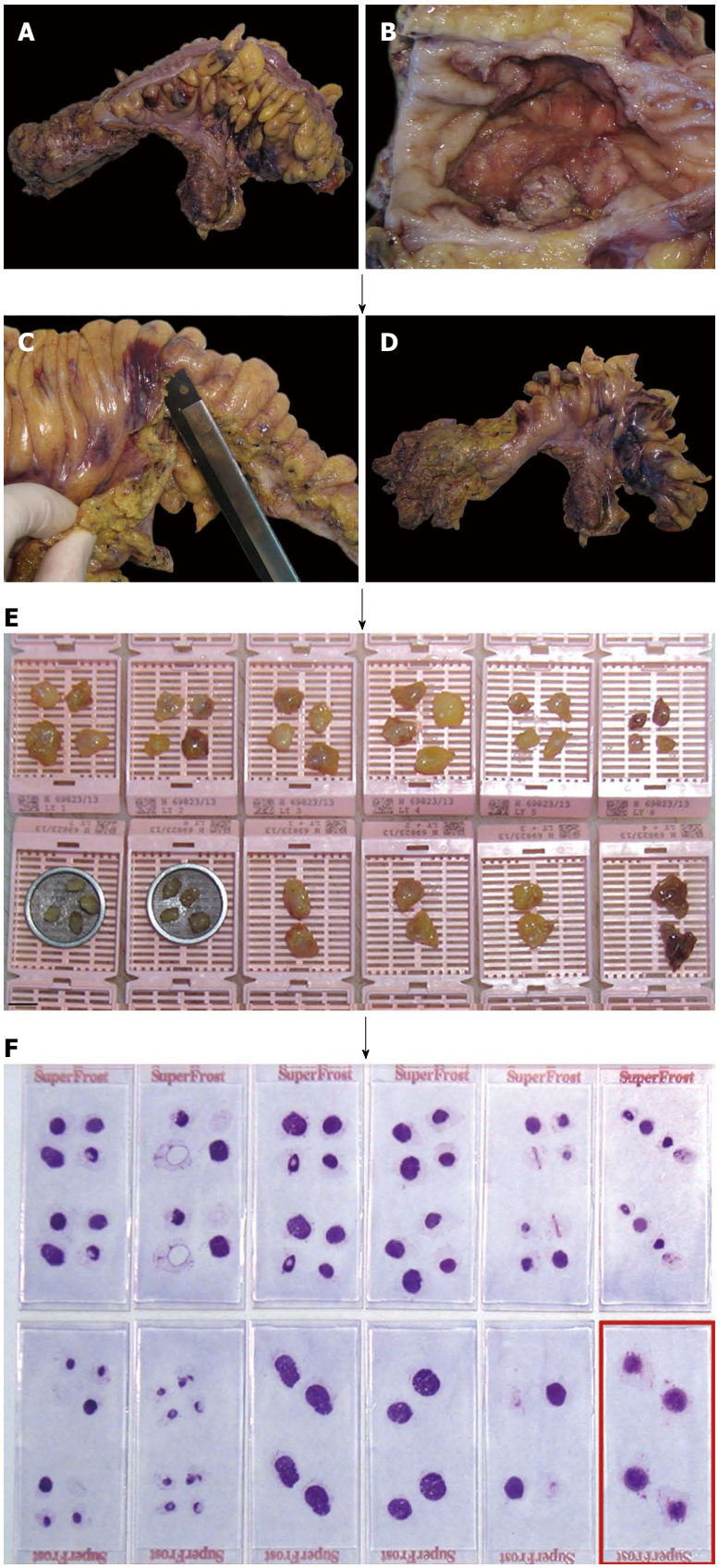

Figure 1 Manual dissection with subsequent histological assessment based on routinely hematoxylin and eosin stained slides is the standard approach in the examination of regional lymph nodes in cancer specimens. A: Rectum cancer specimen of a 56-year-old female; B: Ulcerated primary tumor, measuring 5 cm in largest diameter; C: After preparation of the primary tumor (including the fatty tissue underneath the lesion and the circumferential margin) the remaining perirectal/mesocolic fatty tissue is carefully removed; D: Specimen for subsequent manual lymph node dissection; E: 36 presumed lymph nodes are isolated, of which the largest four are cut into halves and embedded on their own, respectively (lower right); F: 31 lymph nodes are confirmed on hematoxylin and eosin stained slides, one of which with metastatic cancer tissue (encircled).

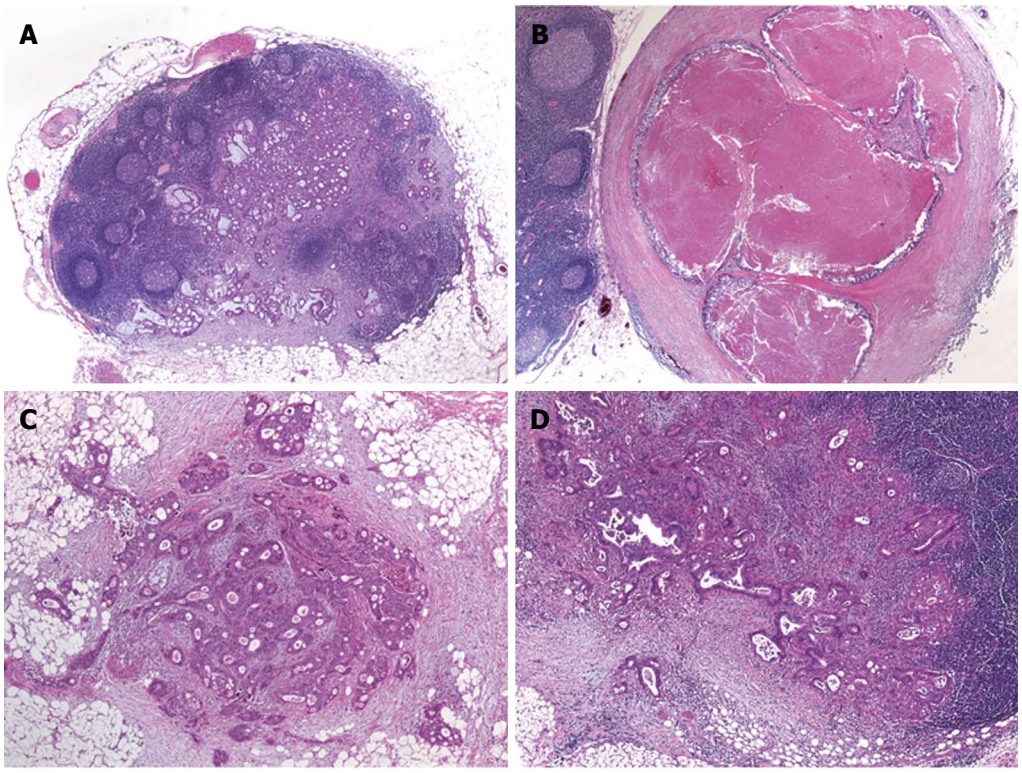

Figure 2 Lymph node metastases and tumor deposits in patients with colorectal cancer. A: Metastatic adenocarcinoma within a mesocolic lymph node [hematoxylin and eosin (HE) original magnification, × 100]; B: Mesocolic lymph node totally replaced by metastatic cancer tissue, note the smooth contour of the lesion (HE, original magnification, × 150); C: Tumor deposit (satellite) within the mesocolic fatty tissue, note the irregular contour of the lesion (HE, original magnification, × 250); D: Mesocolic lymph node metastasis with extracapsular extension of cancer tissue (original magnification, × 250).

strated in two independent populations. Therefore, several national guidelines in Europe still refer to TNM-5 for classification. It is simpler, more reproducible, allows for comparison with preoperative imaging, and is effective and accurate[5]. The potential value of TNM-7 remains to be evaluated in larger prospective studies. The fact that all patients with tumor deposits will now be classified in the node-positive group has raised major concerns. This holds true particular for the evaluation of tumor regression and residual tumor foci after neoadjuvant therapy. In the group of patients who did not receive preoperative treatment, however, staging according to TNM-7 appears to be highly prognostic and possibly superior to TNM-5 and TNM-6[5]. The reproducibility of the definitions given in the latest version may, however, be imperfect. In a recent interobserver variability study of lymph nodes and tumor deposits by Rock *et al*[22], seven gastrointestinal pathologists completely agreed on only 11 of 25 lesions (κ-value 0.48; 95%CI: 0.28-0.67). Top-ranked features for the differentiation of lymph node metastases from tumor deposits included round shape, peripheral lymphocyte rim, peripheral lymphoid follicles, subcapsular sinus, residual lymph node surrounding fibroadipose tissue, and thick capsule. As inconsistency remains even under expert pathologists, it is currently unclear whether the criteria that are available for the distinction of lymph node metastases from tumor deposits are feasible in everyday routine practice performed by general pathologists.

MINIMUM NUMBER OF LYMPH NODES

Adequate assessment of nodal status depends on the total number of retrieved lymph nodes that are available for histological evaluation. A recommendation put forward by Fielding *et al*[23] stated the ideal minimum to be 12 nodes since below this cut-off value there is a high risk of false-negative reporting of lymph node involvement due to inadequate sampling[16]. This recommendation was adopted by the AJCC/UICC TNM system and has been included in various clinical practice guidelines[2-4,24]. The minimum number of lymph nodes that should be assessed ensures adequate staging, prognostication, and accurate treatment, since affected lymph nodes are the primary determinant for the use of adjuvant chemotherapy.

The variability in the number of retrieved lymph nodes remains to be a major problem in patient management since often the recommended minimum number of 12 lymph nodes is not achieved. This may be due to differences in the extent of surgical lymph node removal, the thoroughness of the pathologist in dissecting the cancer specimen, and/or the actual number of regional lymph nodes that is related to tumor location[25,26]. In rectal cancer, the increasing use of neoadjuvant therapy represents another important factor affecting lymph node yield. Under combined chemo-and radiotherapy regional lymph nodes undergo a process of regression. Thus, the recommended number of 12 lymph nodes was

Table 2 Changing definitions of lymph nodes, involved lymph nodes, and tumor deposits in different editions of the American Joint Committee on Cancer/Union for International Cancer Control tumor node metastasis staging system

TNM-5	A tumor nodule greater than 3 mm in diameter in perirectal or pericolic adipose tissue without histological evidence of a residual lymph node in the nodule is classified as regional lymph node metastasis. However, a tumor nodule up to 3 mm in diameter is classified in the T category as discontinuous extension, *i.e.*, T3
TNM-6	A tumor nodule in the pericolic/perirectal adipose tissue without histological evidence of residual lymph node in the nodule is classified in the pN category as a regional lymph node metastasis if the nodule has the form and smooth contour of a lymph node. If the nodule has an irregular contour, it should be classified in the T category and also coded as V1 (microscopic venous invasion) or V2, if it was grossly evident, because there is a strong likelihood that it represents venous invasion.
TNM-7	Tumor deposits (satellites), *i.e.*, macroscopic or microscopic nests or nodules, in the pericolorectal adipose tissue's lymph drainage area of a primary carcinoma without histological evidence of residual lymph node in the nodule, may represent discontinuous spread, venous invasion with extravascular spread (V1/2) or a totally replaced lymph node (N1/2). If such deposits are observed with lesions that would otherwise be classified as T1 or T2, then the T classification is not changed, but the nodule(s) is recorded N1c. If a nodule is considered by the pathologist to be a totally replaced lymph node (generally having a smooth contour), it should be recorded as a positive lymph node and not as a satellite, and each nodule should be counted separately as lymph node in the final pN determination.

TNM: Tumor node metastasis.

reached in only about 20% of cases in large international trials that investigated the benefit of neoadjuvant therapy in rectal cancer. This observation prompted the question whether the insufficient number of lymph nodes is due to the disappearance of the nodes, or just reflects progressive atrophy and fibrosis with subsequent reduction in lymph node size, rendering them undetectable during routine pathological work-up[27].

Due to the fact that the recommended number of nodes is often not reached by traditional manual dissection new technical methods were introduced to facilitate lymph node harvest in the fatty tissue. These include fat clearing methods, methylene blue-assisted lymph node dissection, as well as acetone elution with subsequent compression of adipose tissue ("acetone compression"). The method of methylene blue-assisted lymph node dissection was introduced in 2007 as a cheap and simple tool[28]. The method is based on *ex vivo* intraarterial injection of 15-20 mL of methylene blue solution in the fresh or shortly formalin-fixed resection specimen. After fixing overnight lymph nodes are dissected manually. This technique results in dramatically increased lymph node counts compared to conventional dissection. The effect is particularly evident in rectal cancer patients after neoadjuvant therapy and helps to ensure a sufficient lymph node harvest in these patients. However, according to a recently published study[29], the application of this technique does not seem to be associated with an increased detection of lymph node metastases. In this study, comparing methylene blue assisted dissection with standard dissection, neither the rate of nodal positive cases, nor the rate of pN_2 cases differed between the two groups. The most probable explanation for this finding is the fact that mostly involved lymph nodes are enlarged and therefore easy to find[30].

The acetone elution and compression method was introduced by Basten *et al*[31]. After manual dissection for large palpable lymph nodes (usually > 1 cm in diameter) the mesorectal fat is perforated with a needle roller and transferred to acetone. After elution in acetone, tissue samples are mechanically compressed using a manual squeezing machine, as described in detail by Gehoff *et al*[27]. By this method a reduction of about 90% of mesorectal fat volume is achieved. Specifically, acetone compression facilitates the detection of any tumor deposit in mesorectal and mesenteric fatty tissue and therefore provides a reliable survey of tumor cell deposits including perineural cancer infiltrates, particularly after neoadjuvant therapy[27]. As for methylene blue-assisted lymph node dissection, the total number of harvested lymph nodes markedly increased in that study, the number of positive lymph nodes, however, did not change. From a biological standpoint it is interesting to note that, basically, the number of lymph nodes is independent of pretreatment status[27].

LYMPH NODE RATIO OR ABSOLUTE NUMBER OF INVOLVED LYMPH NODES?

Several studies have demonstrated that simply the analysis of a larger number of lymph nodes results in a survival advantage for patients with stage Ⅱ and Ⅲ disease, while the situation for stage Ⅰ disease is less clear[32-36]. A study by Lykke *et al*[36] demonstrated that in patients with more than 12 nodes, there was a significantly higher proportion of stage Ⅲ disease, indicating that stage migration takes place when high numbers of lymph nodes are harvested. To overcome the dependence on the number of harvested lymph nodes, a ratio-based node staging system has been proposed.

The lymph node ratio, defined as the number of positive lymph nodes divided by the total number of retrieved nodes, has gained increasing attention. A large number of studies showed that the prognostic significance of lymph node ratio is superior to that of the absolute number of involved lymph nodes[6,8,36-44]. Lymph node ratio was identified as an independent predictor of disease-free survival, overall survival, and cancer-specific survival in stage Ⅲ disease. Notably, lymph node ratio remains to be an independent prognosticator even after neoadjuvant therapy, despite reduction of the absolute number of retrieved nodes[45]. The lymph node ratio may thus improve TNM-based prognostic stratification and may help to identify patients at high risk of disease recurrence and/or

progression.

Problems, however, remain, particularly as different cut-off values were applied in the studies that identified lymph node ratio as promising tool. Currently, we do not know which cut-off value is ideal and whether this value may be the best for both colon and rectum cancer. Future prospective studies, applying a data-driven approach are urgently needed to accurately define these cut-off values, as obviously "not one size fits all"[40].

Although the concept of lymph node ratio was developed to generate a prognostic marker that is independent from the number of examined nodes data are still conflicting in this regard. According to Chen *et al*[43], lymph node ratio independently estimates survival, irrespective of the number of nodes examined. In the study by Berger *et al*[38], lymph node ratio was a significant parameter when 10 or more lymph nodes were removed, but not for patients with less than 10 lymph nodes.

EXTRACAPSULAR LYMPH NODE INVASION

Extracapsular lymph node invasion refers to the extension of cancer cells through the nodal capsule into the perinodal fatty tissue (Figure 2D)[46]. This phenomenon has gained considerable attention as prognostic variable in several solid organ tumors, particularly in cancers originating from breast and head and neck region as well as in several gastrointestinal malignancies.

In colorectal cancer, extracapsular invasion has been observed in 18% to 68% of stage Ⅲ tumors[10-13,46]. According to Komuta *et al*[10], extracapsular invasion occurs more likely in lymph nodes that are occupied for more than 50% by cancer cells, compared to lymph nodes with less than 50% occupation. Its occurrence has been related to high pT-classification, high number of involved nodes, and presence of positive distant lymph nodes, which allows the conclusion that extracapsular invasion is more likely to be found in advanced tumor stage[11-13].

The ability of metastatic nodes to recruit degradation factors that permit cancer cells to break through the lymph node capsule reflects the invasiveness and aggressiveness of the primary tumor, even in an immunologically hostile environment[46]. Thus, patients with extracapsular invasion at metastatic sites are at particularly high risk to develop disease progression and distant cancer spread[12,13]. In particular, survival and recurrence rates of patients with extracapsular invasion are significantly worse than those of patients without, and extracapsular invasion has been identified as independent predictor of disease-free and overall survival in patients with node positive cancers[11,12,47,48].

Overall, the detection and, possibly, quantification of extracapsular invasion may help to individualize postoperative treatment strategies by identification of a subgroup of patients with significantly poorer long-term survival and poorer local control who might benefit from intensified adjuvant therapy[46].

SENTINEL LYMPH NODE BIOPSY

The sentinel lymph node, defined as the first lymphatic station within a given lymph drainage area, is considered to be of eminent importance in oncologic practice. Sentinel node detection may be accomplished by injection of blue dye (*e.g.*, methylene blue) or radiotracers near to the tumor. Afterwards the surgeon detects the node by visual inspection or by use of gamma probe or Geiger counter. In clinical practice, sentinel lymph node biopsy has been found to be highly effective in correctly predicting the nodal status in malignant melanoma and breast cancer[49]. Commonly, a frozen section procedure is employed so if neoplasia is detected further lymph node dissection may be performed. If, however, the sentinel node is free of cancer the extent of operation may be kept to a minimum.

Within the last two decades, several investigators aimed to enlarge the field of application and have evaluated sentinel lymph node biopsy in various malignancies[50]. In colorectal cancer, the potential benefit of sentinel lymph node biopsy is different from that of malignant melanoma and breast cancer. Here, the method does not intend to reduce the extent of surgery but aims to identify conditions that might lead to more extensive surgical lymph node dissection. Another purpose is to establish more accurate lymph node staging in order to identify patients at risk for disease recurrence and/or progression[51].

According to a recent meta-analysis[52], the pooled sentinel node identification rate is approximately 90% in patients with colorectal cancer, with a significantly higher rate in studies including more than 100 patients or studies using an *ex vivo* approach. The pooled sensitivity of the procedure is approximately 70%. Subgroups with significantly higher sensitivity could be identified. These include individuals with $\geqslant$ 4 sentinel nodes identified (*vs* individuals < 4 nodes), colonic location (*vs* rectal location), and early, *i.e.*, $pT_{1/2}$ carcinomas (*vs* advanced, *i.e.*, $pT_{3/4}$ carcinomas).

How sentinel lymph node biopsy may be successfully incorporated in routine practice has recently been illustrated in a study by Saha *et al*[53]. The authors investigated 192 patients undergoing surgery for colon cancer and identified aberrant drainage, *i.e.*, drainage outside the standard resection margin requiring change of the extent of operation, in 22% of patients. Notably, nodal positivity was higher in patients undergoing change of operation (62%) compared to those undergoing only standard oncologic resection (43%).

Major drawbacks remain to be the still imperfect detection rate and the comparably low sensitivity for the identification of nodal disease. The detection rate is significantly influenced by several patient-and disease-specific factors, the most important of which being body mass index, center experience, and learning curve[49]. The considerably high false-negative rate to identify node-positive patients may be explained by aberrant drainage sites and the presence of skip lesions. It is known that skip lesions

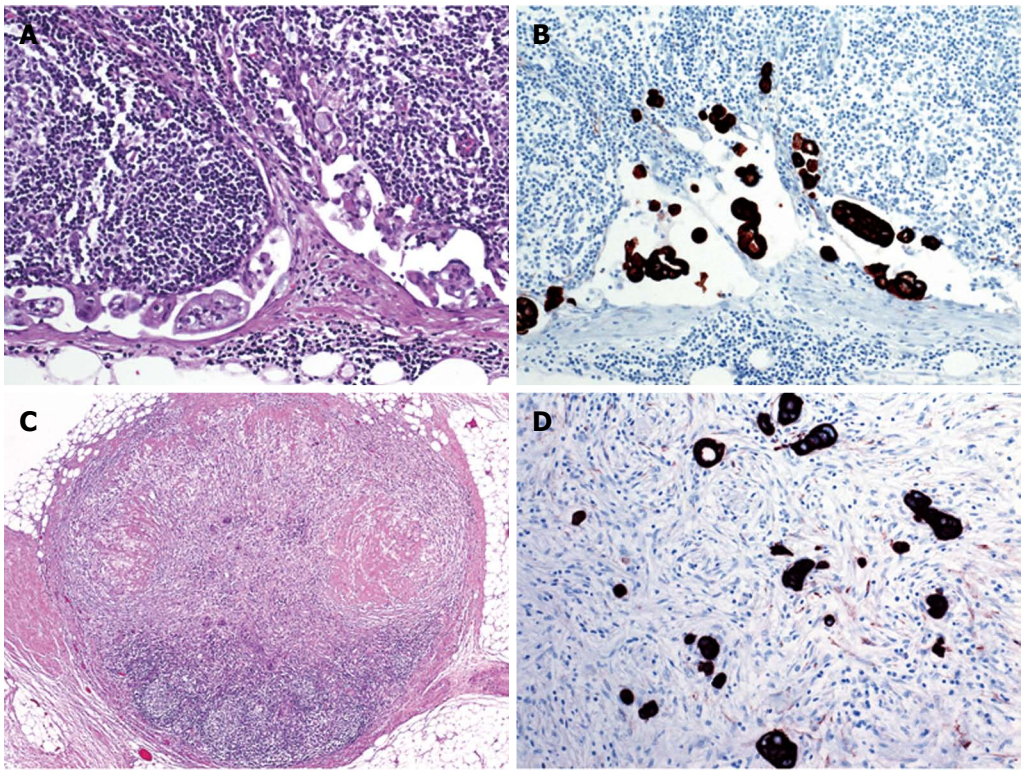

Figure 3 Value of immunohistochemistry in the evaluation of lymph nodes from patients with colorectal cancer. A: Micrometastasis in the subcapsular sinus of a mesocolic sentinel node evaluated by standard hematoxylin and eosin (HE) staining (original magnification, × 400); B: Micrometastasis in the subcapsular sinus of a mesocolic sentinel node evaluated by immunohistochemistry using an antibody preparation directed against pankeratin (serial section to A, original magnification, × 400); C: Atrophic perirectal lymph node with marked fibrosis after neoadjuvant treatment (original magnification, × 100); D: Identification of residual cancer cells by pankeratin immunostaining (original magnification, × 400).

occur when lymphatics are obstructed by tumor. Retter *et al*[54] showed that in 63% of their false negative tumors, lymphatic and venous invasion by cancer cells was present.

The extent of the pathological work-up is another major factor with significant impact on the performance and clinical significance of the sentinel node biopsy concept. According to the meta-analysis cited above, adding step sectioning and immunohistochemistry, *e.g.*, using antibodies directed against pankeratin (Figure 3), to the pathological work-up resulted in a mean upstaging in 18.9% (range 0%-50%). True upstaging defined as micrometastases [pN1(mi)] rather than isolated tumor cells [pN0(i+)] occurred in 7.7%[52]. The optimal technical method how sentinel lymph nodes should be evaluated still has to be defined. Several papers have addressed this topic, the three most relevant will be referred to in detail.

In the study by Bembenek *et al*[49], a total of 141 of 186 patients classified as nodal negative by routine HE staining underwent step sectioning and immunohistochemical analysis for pankeratin (MNF116) of their sentinel lymph nodes. Thirty of these patients revealed micrometastases (n = 7) or isolated tumor cells (n = 23), resulting in an overall upstaging rate of 30 of 141 (21.3%). In the clinically important subgroup of stage II patients, upstaging occurred in 24.2% (21 of 91).

In the study by van der Zaag *et al*[51], three serial sections (cut at 500 μm intervals) of all 908 lymph nodes from 58 patients with pN0 carcinomas (according to standard evaluation on HE stained slides) were examined with three different antibodies [directed against pankeratin (Cam5.2), keratin 20, and Ber-EP4]. The examination revealed occult tumor cells in 33% (19 of 58) of histologically pN0 patients (12% micrometastases and 21% isolated tumor cells). Occult tumor cells were predominantly found in sentinel nodes with an overall sensitivity of sentinel mapping for occult tumor cells of 88%.

In the study by Märkl *et al*[55], applying methylene blue injection in an *ex vivo* approach lymph node metastases were found in 20 of 47 (43%) cases with skip metastases occurring in four of them. Performing three additional HE step sections and immunohistochemical staining for pankeratin (MNF116) in sentinel lymph nodes and all other lymph nodes, resulted in true upstaging (N0N1mi) in 1 of 23 cases (4%).

MOLECULAR ANALYSIS OF LYMPH NODES - A FEASIBLE APPROACH?

The identification of lymph node involvement is the most important factor to predict outcome and qualify affected patients for adjuvant chemotherapy[55]. Manual dissection of fatty tissue and histopathology based on HE stained sections remain to be the standard approach in pathological lymph node evaluation.

Table 3 Markers for molecular lymph node staging

Keratin 20
Keratin 19 (including one-step nucleic acid amplification technique)
Mucin apoprotein 2
Guanylyl cylase C
Carcinoembryonic antigen
CEACAM6
CEACAM1-S
CEACAM1-L
CEACAM7-1
CEACAM7-2
c-Met
K-ras mutation
Estrogen receptor promoter methylation

This may, however, lead to underestimation of disease and understaging of patients. About 30% of the patients with histopathology-negative lymph nodes (AJCC/UICC TNM stages Ⅰ and Ⅱ) develop recurrent and/or progressive disease, likely associated with undetected metastatic deposits[15,56-59]. As shown above, the use of additional step sections and immunohistochemistry may improve the identification of positive lymph nodes. Of note, many patients initially staged lymph node-negative, who experienced disease recurrence had isolated tumor cells and/or micrometastases after advanced evaluation[60]. A major limitation of the histological examination is the fact that only a small portion of the lymph node, usually the section(s) with the largest cut surface, is assessed leaving most parts of the nodes uninspected[61].

As current techniques for nodal examination may be inadequate for the detection of micrometastases and/or isolated tumor cells, molecular analysis of lymph node tissue has been introduced as additional tool in the work-up of cancer patients. The identification of minimal disease in lymph nodes by molecular techniques may help to identify patients at high risk for recurrence and/or progression, who could benefit from adjuvant therapy[62]. The following features are relevant: (1) no expression of the respective marker in immune cells; (2) no or weak downregulation in tumors compared to normal tissue; and (3) relatively high and constant expression in tumor tissue irrespective of tumor stage[63]. Several molecular markers have been applied (Table 3). In the following we will refer to some of them in detail.

Keratin 20 (K20) is constitutively expressed in intestinal epithelia and is the most important keratin subtype expressed in colorectal cancer. It can be found in more than 90% of primary tumors. Immunoreactivity in metastatic tissues is known to match well with that of corresponding primary tumors, with high concordance for lymph node and distant metastases, respectively[64]. The significance of quantitative real-time polymerase chain reaction (RT-PCR) for the detection of K20 mRNA in regional lymph nodes of cancer patients has been investigated by several groups, mainly in sentinel node biopsies[15,57,65-71]. In general, these studies demonstrated a higher sensitivity of molecular analysis compared to standard evaluation based on HE stained slides and also compared to advanced evaluation applying immunohistochemistry.

MUC2 apoprotein, which is secreted from non-neoplastic intestinal goblet cells and is expressed in the majority of colorectal cancers, has been introduced as another promising marker[15,63,72,73]. Some groups investigated carcinoembryonic antigen[63,68,69,72,74], while others referred to guanylyl cylase C (GCC)[58,68,75]. GCC is a receptor for bacterial enterotoxins and the paracrine ligands guanylin and uroguanylin and is expressed selectively by intestinal epithelium. Comparable to mucin apoprotein 2 (MUC2), the expression of GCC is preserved throughout the transition from adenoma to carcinoma in colorectal tissues[56,58]. Most recently, so-called one-step nucleic acid amplification (OSNA) has been introduced to detect keratin 19 (K19) mRNA as a surrogate for lymph node metastasis. K19 is expressed in many types of cancer, albeit in varying frequencies. OSNA is based on reverse transcription-loop-mediated isothermal amplification to amplify K19 mRNA[59,61,76].

All these techniques allow the examination of the entire lymph node, thereby overcoming the problem of sampling bias due to insufficient analysis of material in the standard histological approach. This may lead to improved staging and better selection of patients for adjuvant chemotherapy. More importantly the molecular detection of tumor cells in regional lymph nodes has been associated with disease recurrence and poor survival in node-negative colorectal cancer[14,77].

Problems, however, remain. The value of quantitative RT-PCR assays for the detection of occult tumor cells in regional lymph nodes relies on the balance between sensitivity and specificity in order to minimize the occurrence of false-positive or false-negative results[78]. None of the markers are really specific. K19 has been used as a molecular marker in a variety of studies dealing with several types of cancer, including colorectal cancer. Doubt has arisen about the tissue specificity of K19 gene expression. Already in 1996, Gunn *et al*[79] noted *K19* gene expression in 34 of 40 lymph nodes from patients who underwent bowel resection for benign disease. The reasons for the observed false-positivity rate are not entirely clear. In addition to simple contamination or dissemination of tumor cells and/or tumor cell fragments *via* the lymphatics during the procedure, amplification of K19 pseudogenes may play a role[78]. Finally, Bustin *et al*[68] detected K20, carcinoembryonic antigen, and GCC mRNA in 47%, 89% and 13% of 149 lymph nodes, respectively from patients with benign disease indicating that K19 is not the only marker for which specificity problems remain to be solved.

Nevertheless, the molecular approach has opened new options concerning the diagnosis of isolated tumor cells and micrometastases in patients with histopathology-negative lymph nodes[57]. Benefits of the molecular approach have to be weighed against potential drawbacks. A major reason for controversy is the lack of standardization of molecular analyses hampering comparison of different studies as well as inclusion of molecular techniques into

routine practice[57]. According to current practice guidelines, AJCC/UICC stage Ⅲ patients receive adjuvant treatment. This strategy results in significantly improved outcome when nodal disease is proven histologically. However, it is currently not entirely clear whether the patients with nodal disease proven on a molecular level experience similar benefits if chemotherapy is given.

CONCLUSION

Lymph node staging is a major prognostic factor in colorectal cancer and remains to be the most important criterion to select patients for adjuvant treatment. Changing definition of lymph nodes, involved lymph nodes, and tumor deposits in different editions of the AJCC/UICC TNM system have influenced the significance of lymph node staging in the past. The standard approach for lymph node evaluation is based on manual dissection and histological evaluation of HE stained slides. Methylene blue injection and fat clearing methods increase lymph node harvest in cancer specimens. Adding step sectioning and immunohistochemistry to the pathological work-up may result in higher accuracy of histological diagnosis. The clinical value of more recent techniques, such as sentinel lymph node biopsy and molecular analysis of lymph nodes tissue still remains to be defined.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Different standards for healthy screenees than patients in routine clinics?

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Abstract

Less than 5% of colorectal adenomas will become malignant, but we do not have sufficient knowledge about their natural course to target removal of these 5% only. Thus, 95% of polypectomies are a waste of time exposing patients to a small risk of complications. Recently, a new type of polyps, sessile serrated polyps, has attracted attention. Previously considered innocuous, they are now found to have molecular similarities to cancer and some guidelines recommend to have them removed. These lesions are often flat, covered by mucous, not easily seen and situated in the proximal colon where the bowel wall is thinner. Thus, polypectomy carries a higher risk of perforation than predominantly left-sided, stalked adenomas - and we do not know what is gained in terms of cancer prevention. Screening is a neat balance between harms and benefit for presumptively healthy participants not interested in risk exposure to obtain confirmation of being healthy. The situation is quite different for patient worried about symptom. Thus, the standards set for evidence-based practice may be higher for screening than for routine clinics - a mechanism which may benefit patients in the long run.

Key words: Colonoscopy; Screening; Quality assurance; Standards

Core tip: There is a basic difference in incitements to attend for screening when you are healthy and for routine clinics when you are ill. This article points out logical mechanisms which may set standards for screening higher than for routine clinics, but this may prove to be of benefit for clinical services and patients in the long run. This is highlighted by sessile serrated polyps which were previously classified as innocuous hyperplastic polyps. Recent guidelines now recommend polypectomy of these lesions for cancer prevention, but we do not know the benefit gained - only the increased risk of perforation by polypectomy.

Hoff G. Different standards for healthy screenees than patients in routine clinics? *World J Gastroenterol* 2013; 19(46): 8527-8530 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i46/8527.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i46.8527

MECHANISMS FAVOURING DIFFERENT STANDARDS

We know that there are endoscopist-dependent variations in colonoscopy performance - whether this service is provided in routine clinics or screening[1-4]. Quality assurance (QA) initiatives driven by health care providers may be half-hearted - particularly when demands for colonoscopy outnumber available capacity and reducing unacceptable waiting lists is first priority. Within the European Union, however, it has been stated explicitly for screening that only organized screening that can be evaluated is to be

accepted, "performance indicators should be monitored regularly" and the population should be protected from "poor-quality screening"[5]. Independent of such policy statements for QA which may have its counterparts in clinical non-screening services in many countries, client or patient-driven QA may have a stronger impact in screening programmes than in routine clinics. The option of not attending if the quality is sub-standard is both more realistic and a dreadful threat to screening programmes compared to routine clinical services. Whichever colorectal cancer (CRC) screening method is used, high attendance rates are crucial for the success of any screening programme - "the best screening test is the one that gets done"[6].

There is a basic difference between screening participants and patients. Screenees are presumptively healthy individuals who seek confirmation that they are just that - healthy. Patients have symptoms and disease for which they seek whatever help may be offered. This means that patients may be more willing to accept some risk of complications, harms and discomfort to be cured. It is reasonable that screenees are not willing to subject themselves to risks and discomfort to obtain confirmation of being healthy.

Screening participants: Presumptively healthy seeking confirmation of being healthy. Not willing to take risks to obtain this confirmation. They request documentation of benefits and harms - "what is in it for me?"

Patients: They have symptoms or known disease for which they seek whatever help they may be offered. It may be a matter of clinging to a hope of cure with great willingness to pay and few questions asked on documentation of effect - "please, just do something!"

Since high attendance rates are crucial for screening programmes, it is important to understand the reasons for non-attendance. This is far from a primary issue in routine clinics serving patients. In focus groups addressing CRC screening, both representatives of target populations and family doctors have expressed scepticism to screening, questioning the evidence of its effectiveness[7]. To meet these critics, facts about risks and benefits and defining fields of uncertainty must be produced and made accessible in a trustworthy and understandable format to provide a basis for informed decision-making by members of the target population[8,9]. This is quite a different exercise from campaigning for screening by appealing to fear, guilt and personal responsibility - methods that may have been used too frequently in the short history of screening to improve attendance[10]. Such campaigning will only tear down any trustworthiness there may have been. There should be a strong incitement to provide high-degree level of evidence to support (or discard) screening - evidence that can withstand scepticism and critics generated by poor-level evidence and over-selling screening services[10].

SIZE OF THE PROBLEM AND THE HIGH INTENTIONS OF DOING GOOD

On a worldwide basis, there are more than 1.2 million new cases of CRC diagnosed annually with prospects of 5-year survival for 50%-60% of patients[11,12]. Symptoms often appear late and they are unspecific - mimicking common and more trivial conditions like haemorrhoids and irritable bowel. Although progress is being made on treatment of advanced CRC, new drugs are driving costs, but the best bet for cure remains early diagnosis and surgery. Both to get at the cancer at an early, asymptomatic stage to save lives and suffering - and to save costs for treatment of advanced disease[13], CRC screening is recommended in several countries[14]. There are several screening methods, but only fecal occult blood tests (FOBT) and flexible sigmoidoscopy (FS) have been subjected to randomized trials (RCT) with long-term follow-up[15-18]. By intention-to-treat analyses, FOBT screening reduces CRC mortality by 15%-18% with no effect on CRC incidence. FS screening reduces mortality by 28% and incidence by 18%[18]. Intuitively, colonoscopy screening should be twice as good as FS ("half-way colonoscopy") combining "gold standard" sensitivity for CRC and polyp detection with tissue sampling and removal of CRC precursor lesions (polyps). There are RCTs on colonoscopy underway, but results are not expected for many years[19,20]. Retrospective studies, however, have suggested that colonoscopy screening may not be as effective as expected in reducing right-sided CRC[21]. It has been suggested that right-sided (proximal) sessile serrated polyps, which are easily overlooked and share molecular similarities to CRC, may represent an additional polyp-carcinoma pathway similar to the traditional adenoma-carcinoma pathway[22]. This may explain poorer results than expected for colonoscopy in reducing the burden of right-sided CRC. When the trials on FOBT and FS screening were done, endoscopists and pathologists largely considered sessile serrated polyps to be hyperplastic and non-neoplastic with no intrinsic potential to develop into CRC. Changing to go aggressively for these right-sided sessile lesion has its implications (*e.g.*, higher risk of perforation at polypectomy) and we really do not know what there is to be gained - *i.e.*, we cannot quantify expectations of a reduced risk of CRC.

OVERTREATMENT WITH A FEAR OF NOT DOING ENOUGH

Screening is a neat balance between benefits and harms - benefit for the few (those few discovered to have asymptomatic CRC or advanced adenoma) *vs* inconvenience and potential risks for the many (all other participants). Providing data on CRC mortality and/or incidence reduction is a prerequisite before implementing screening

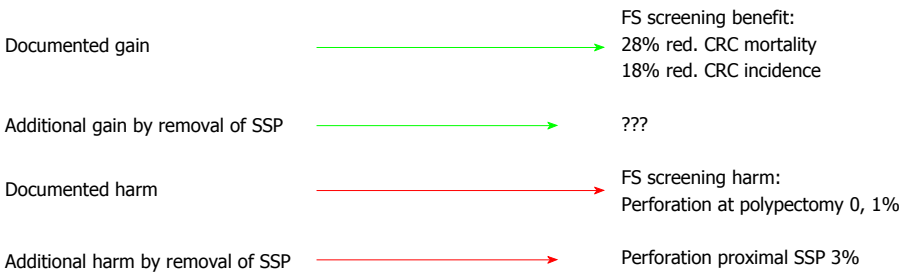

Figure 1 Overtreatment with a fear of not doing enough. Introducing uncertainty in the balance between benefits and harms of flexible sigmoidoscopy (FS) screening by adding systematic removal of proximal sessile serrated polyps (SSP) associated with 3% risk of perforation for these lesions[23] and unknown benefit compared to 0.1% risk of perforation[27] and known benefit. CRC: colorectal cancer.

programmes[5], but the target population should also receive valid information on the downsides of screening, like the risk of perforation and bleeding when polypectomy is recommended. We now have long-term results from RCTs on FOBT and FS screening based on the standards used in the trials, including work-up colonoscopies and surgical treatment, and we can provide the target population with information of what is to be gained in terms of mortality and incidence reduction and the risks involved with endoscopy, polyp removal and surgery when required. This is very much a satisfactory level of practicing "evidence-based medicine".

Our current practice of polyp treatment and surveillance is largely based on consensus guidelines. If we change our practice in screening programmes from the standards used in trials preceding the programmes, we do this because we believe such adjustments are for the good. The intentions may be the best, but is the evidence up to standards required for the target population to feel it worthwhile attending for screening? RCTs on FS screening give 18% reduced risk of CRC with a 0.04% risk of perforation and 0.1% risk of perforation at work-up colonoscopy[18]. But - more meticulous search and removal of proximal sessile serrated polyps may involve a risk of 3% for severe complications (perforation and bleeding) for these lesions[23] with no evidence of what to be gained (Figure 1). This is a level of uncertainty that may not be questioned by patients, but more likely tilt the decision of the potential screenee towards not attending.

Overdiagnosis and overtreatment of cancer is a recent issue that has emerged from screening activity - not from routine clinical work[24]. For CRC, we know that more than 95% of polypectomies are a waste of time involving unnecessary risks, but we do not know which 5% to go for. After more than 120 years of the adenoma-carcinoma sequence theory[25], we do not know the natural history of adenomas. We can say very little about future risk of CRC in a polypectomized adenoma - had it not been removed. It is desirable with better definition and targeting of high-risk polyps to be removed and low-risk lesions to be ignored at colonoscopy. It is hard to see how this knowledge-gap can be filled without accepting prospective studies on in-situ polyps. With a low risk for complications at polypectomy, this may not be acceptable. Moving towards more aggressive interventions without knowing the magnitude of expected benefit, we may eventually reach a line when screening either is to be stopped or modified due to complications. At that point in time the problems of overtreatment may become so pronounced that in-situ research with all possible security measures may be accepted. There may be more at stake for screening programme providers and participants (screenees) on this issue than for patients, and it may be that comparative effectiveness research (CER) within screening programmes[26] may provide possibilities to fill this and other knowledge-gaps - also for the benefit of clinical practice. Among 45 original publications on the main study and sub-studies published so far from the Norwegian Colorectal Cancer Prevention trial (NORCCAP) there were several findings of transfer value to routine clinical practice - particularly on endoscopy technique and technology (listed in www.kreftregisteret.no/norccap).

CONCLUSION

There is a basic difference in incitements to attend for screening when you are healthy and for routine clinics when you are ill. This may be more clearly brought forward by an increasing demand for patients and clients to have a say in QA of health care provisions - both in screening and routine clinics. There are logical mechanisms which may set standards for screening higher than for routine clinics, but this may prove to be of benefit for clinical services and patients in the long run.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Immunotherapy for colorectal cancer

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Abstract

The incidence of colorectal cancer (CRC) is on the rise, and the prognosis for patients with recurrent or metastatic disease is extremely poor. Although chemotherapy and radiation therapy can improve survival rates, it is imperative to integrate alternative strategies such as immunotherapy to improve outcomes for patients with advanced CRC. In this review, we will discuss the effect of immunotherapy for inducing cytotoxic T lymphocytes and the major immunotherapeutic approaches for CRC that are currently in clinical trials, including peptide vaccines, dendritic cell-based cancer vaccines, whole tumor cell vaccines, viral vector-based cancer vaccines, adoptive cell transfer therapy, antibody-based cancer immunotherapy, and cytokine therapy. The possibility of combination therapies will also be discussed along with the challenges presented by tumor escape mechanisms.

Key words: Colorectal cancer; Cytotoxic T lymphocyte; Dendritic cell; Immunotherapy; Vaccine

Core tip: The prognosis for patients with recurrent or metastatic colorectal cancer (CRC) is extremely poor. Immunotherapy may be effective for treating CRC patients and/or preventing relapse. The immunotherapeutic approaches for CRC, including peptide vaccines, dendritic cell-based cancer vaccines, whole tumor cell vaccines, viral vector-based cancer vaccines, adoptive cell transfer therapy, antibody-based cancer immunotherapy, and cytokine therapy have been demonstrated. The blockade of multiple immune regulatory checkpoints combined with immunotherapy and/or conventional chemotherapy may be effective in treating patients with advanced CRC.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in men (accounting for 10.0% of all cancers) and the second most common cancer in women (accounting for 9.4% of all cancers) worldwide. Additionally, CRC is the fourth most common cause of cancer-related death[1]. Optimization of surgical resection for patients with localized disease has dramatically improved 5 year and 10 year survival rates. The prognosis for patients with resectable tumors depends on the disease stage. CRC patients with distant metastasis have a 12% survival rate[2], and more than 20% of CRC patients have metastatic disease at the time of diagnosis[3,4]. Moreover, despite the fact that 80% of CRC patients with localized disease demonstrate complete macroscopic clearance of the tumor by surgery, 50% of CRC patients will relapse due to the presence of micro-metastasis at the time of surgery[5]. Chemotherapy is approved for the treatment of regionally metastatic CRC, but it shows only modest efficacy and is ineffective against distant metastases[6]. The prognosis for patients with advanced disease remains unfavorable due to the frequency of recurrence, distant metastasis, and resistance to chemotherapy. Thus, novel treatment modalities are needed. Interestingly, tumors that develop chemotherapy or radiation resistance are still suitable targets for immunotherapy[7-10]. Therefore, cancer immunotherapy may be effective for treating CRC patients and/or preventing relapse.

ANTITUMOR IMMUNE RESPONSES

T cells

Tumor cells degrade endogenous and exogenous tumor-associated antigens (TAAs) into short peptides (usually 8-10 amino acids) and present them on the cell surface in the context of major histocompatibility complex (MHC) class I molecules. T cell receptor (TCR) interaction with the complex of peptides and MHC class I molecules on tumor cells is a critical event in the T cell-mediated antitumor immune response. T cells that express the αβ TCR generally express CD4+ (helper T cells) or CD8+ (cytotoxic T cells) lineage markers[11]. In particular, CD8+ T cells recognize peptides (usually 8-10 amino acids) derived from TAAs bound by MHC class I molecules on tumor cells. Thus, immunotherapy may promote cancer cell killing by eliciting antitumor immune responses by recognizing specific TAAs on tumor cells.

To induce antigen-specific CD8+ cytotoxic T lymphocytes (CTLs), peptides derived from TAAs must be presented on the surface of antigen presenting cells (APCs) in the context of MHC class I molecules. In contrast, CD4+ T cells recognize peptides (usually 10-30 amino acids) in association with MHC class Ⅱ molecules on APCs and enhance the persistence of antigen-specific CD8+ CTLs through secretion of interleukin (IL)-2 and interferon (IFN)-γ[12]. Therefore, the interaction of the αβ TCR with complexes of peptides and MHC class I and class Ⅱ molecules on APCs is a central event in cancer immunotherapy. The αβ TCR expressed by CD8+ CTLs recognizes MHC class I -peptide complexes on tumor cells and leads to tumor cell killing through effector molecules such as perforin and granzyme B[13]. Moreover, there is increasing evidence that CD4+ T cells play a more direct role in generating efficient antitumor immunity beyond simply assisting[14]. Therefore, effective antitumor responses depend on the presence and function of T cells that recognize and eliminate tumor cells[14,15].

A unique subset of human T cells expresses the TCR-γδ. Human γδT cells include several subsets of cells defined by their TCR. The most common subset of TCR-γδT cells in circulating blood express the Vγ9Vδ2 receptor[16,17]. Although cancer immunotherapy strategies primarily focus on activation of these MHC-restricted T cells, γδT cells and αβT cells share certain effector functions such as cytokine production and potent cytotoxic activity. However, they recognize different sets of antigens, usually in a non-MHC-restricted fashion[16,18]. Thus, T cells can attack tumors in their HLA-unrestricted cytotoxic capacity, as well as by secreting cytokines. Indeed, tumor-infiltrating γδT cells have been detected in a broad range of cancers, including CRC[19]. Importantly, activated γδT cells can kill cells from metastatic renal cell carcinomas, mammary carcinomas, prostate carcinomas and colorectal carcinomas, while sparing normal, untransformed cells[18,19].

Natural killer cells

Natural killer (NK) cells are component of innate immunity responses to tumor cells[20]. NK cells can rapidly kill certain target cells, including tumor cells with down-regulated MHC class I molecules[21]. Thus, NK cells play a critical role in antitumor immunity. NK cells recognize tumor cells *via* cellular stress or DNA damage signals[22]. Activated NK cells directly kill target tumor cells through several mechanisms, including[23]: (1) cytoplasmic granules such as perforin and granzyme B[24]; (2) tumor necrosis factor-related apoptosis-inducing ligand and Fas ligand (FasL)[25,26]; (3) effector molecules such as IFN-γ and nitric oxide (NO)[24,27]; and (4) antibody-dependent cellular cytotoxicity (ADCC)[28]. NK cell activators (IL-2, IL-12, IL-15, and IL-18), have also been validated in preclinical cancer models[23].

Dendritic cells

Dendritic cells (DCs) are potent APCs that have been used in cancer vaccines due to their ability to initiate antitumor immune responses[12]. DCs are characterized by expression of MHC class Ⅰ, class Ⅱ, and costimulatory molecules (B7, ICAM-1, LFA-1, LFA-3, and CD40)[29-31].

These molecules function in concert to generate a network of secondary signals essential for reinforcing the primary antigen-specific signal in T-cell activation[29-31]. DCs process endogenously synthesized antigens into antigenic peptides, which are presented on the cell surface in MHC class I -peptide and recognized by the αβ TCR on naïve CD8+ T cells[12]. DCs can also capture and process exogenous antigens, which are then presented by MHC class I molecules through an endogenous pathway in a process known as "cross-presentation"[32]. Moreover, exogenous antigens from the extracellular environment are also captured by DCs and delivered to the endosomal/lysosomal compartment, where they are degraded to antigenic peptides by proteases and peptidases. These antigens then complex with MHC class Ⅱ for recognition by the αβ TCR of naïve CD4+ T cells[12]. Efficient antigen presentation by MHC class I and class Ⅱ on DCs is essential for evoking tumor-specific immune responses[33]. Mature DCs are significantly better at processing and presenting MHC-peptide to the TCR and inducing CTLs due to higher expression of MHC class I and class Ⅱ and costimulatory molecules[33].

Immature DCs reside in peripheral tissues where they take up and process antigens that are degraded to peptides. These peptides are then bound to MHC class I molecules for presentation to CD8+ CTLs or bound to MHC class Ⅱ molecules for presentation to CD4+ T helper (Th) cells. Differentiation of the immature DCs into mature DCs is triggered by molecular stimuli that are released in response to tissue disturbance and local inflammatory responses caused by pathogens[34]. After antigen uptake and stimulation by the inflammatory response, immature DCs in the peripheral tissues undergo a maturation process characterized by the up-regulation of MHC class I and class Ⅱ and costimulatory molecules, the up-regulation of chemokine receptors such as CCR7, and the secretion of cytokines such as IL-12[34,35]. The mature DCs migrate to secondary lymphoid organs, where they present antigens to CD4+ and CD8+ T cells through the MHC class I and class Ⅱ pathways, respectively[12,34]. Therefore, the aim of immunotherapy is to simultaneously activate CD8+ CTLs (which recognize TAA) and CD4+ Th cells.

Immune suppressive cells

CD4+ Th cells are critical for inducing and regulating immune responses. Immune homeostasis is primarily controlled by two distinct helper T cell subsets, Th1 and Th2 cells[36]. Th1 cells secrete IFN-γ, which can further sensitize tumor cells to CTLs by inducing the up-regulation of MHC class I molecule expression on tumor cells and antigen-processing machinery in DCs[12]. Th2 cells secrete type Ⅱ cytokines such as IL-4 and IL-10 that enhance humoral immunity (the antibody-based antitumor response)[12]. Importantly, tumor cell-derived soluble factors such as transforming growth factor-β (TGF-β) and IL-10 induce tolerance by promoting the expansion of the CD4+α-2R (CD25)+ forkhead box P3 (Foxp3)+ natural Treg subset[37]. Induced Tregs (CD4+CD25+Foxp3-) secrete TGF-β and IL-10 and suppress Th1 and Th2 phenotypes[38,39]. Therefore, Tregs play a pivotal role in tumor progression and the suppression of antitumor immunity.

The cancer microenvironment consists not only of cancer cells but also stromal cells such as cancer-associated fibroblasts, tolerogenic DCs, myeloid-derived suppressor cells, immunosuppressive tumor-associated macrophages (TAMs), and Tregs. These immune suppressive cells secrete vascular endothelial growth factor (VEGF), IL-6, IL-10, TGF-β, soluble FasL, and indolamine-2,3-dioxygenase (IDO)[40], which inhibit antitumor immunity by various mechanisms, including depletion of arginine and elaboration of reactive oxygen species (ROS) and NO. Moreover, the tumor microenvironment promotes the accumulation of Tregs that suppress CD8+ CTL function due to the secretion of IL-10 or TGF-β from Tregs and tumor cells[40] (Figure 1).

IMMUNOTHERAPY

Immunotherapy is an active therapeutic approach designed to trigger the immune system to respond to tumor-specific antigens and attack tumor cells. Immunotherapy strategies include the use of peptides derived from TAAs, whole tumor cells, *in vitro*-generated DCs, or viral vector-based cancer vaccines (Table 1).

Peptide vaccines

A peptide vaccine is based on the identification and synthesis of epitopes, which can induce TAA-specific antitumor immune responses. CRC cells express TAAs such as carcinoembryonic antigen (CEA)[41,42], mucin 1[41-43], epidermal growth factor receptor (EGFR)[44], squamous cell carcinoma antigen recognized by T cells 3 (SART3)[45], β-human chorionic gonadotropin (β-hCG)[46], Wilms' Tumor antigen 1 (WT1)[47], Survivin-2B[48], MAGE3[49], p53[50], or mutated KRAS[51], which are potential targets for immunotherapy. Peptide vaccines targeting these TAAs may be a useful approach for immunotherapy in CRC patients.

Peptide vaccines are simple, safe, stable, and economical. Multiple MHC class I -binding peptides have been identified and tested for immunogenicity. Several peptide vaccines for CRC have been tested in phase I clinical trials. Fifteen patients with advanced or recurrent CRC expressing survivin were vaccinated with a peptide derived from HLA-A*2402-restricted epitopes[48]. In 6 patients, tumor marker levels (CEA and CA19-9) decreased transiently during the survivin-2B peptide vaccination. Moreover, in phase I trial of peptide-cocktail vaccines derived from ring finger protein 43 (RNF43) and translocase of the outer mitochondrial membrane 34 (TOMM34), 8 of 21 patients exhibited antigen-specific CTL responses to both RNF43 and TOMM34, and 12 patients exhibited CTL responses to one of the peptides only[52]. The patients who did not demonstrate any CTL responses had the lowest survival rates. By vaccination with a 13-mer

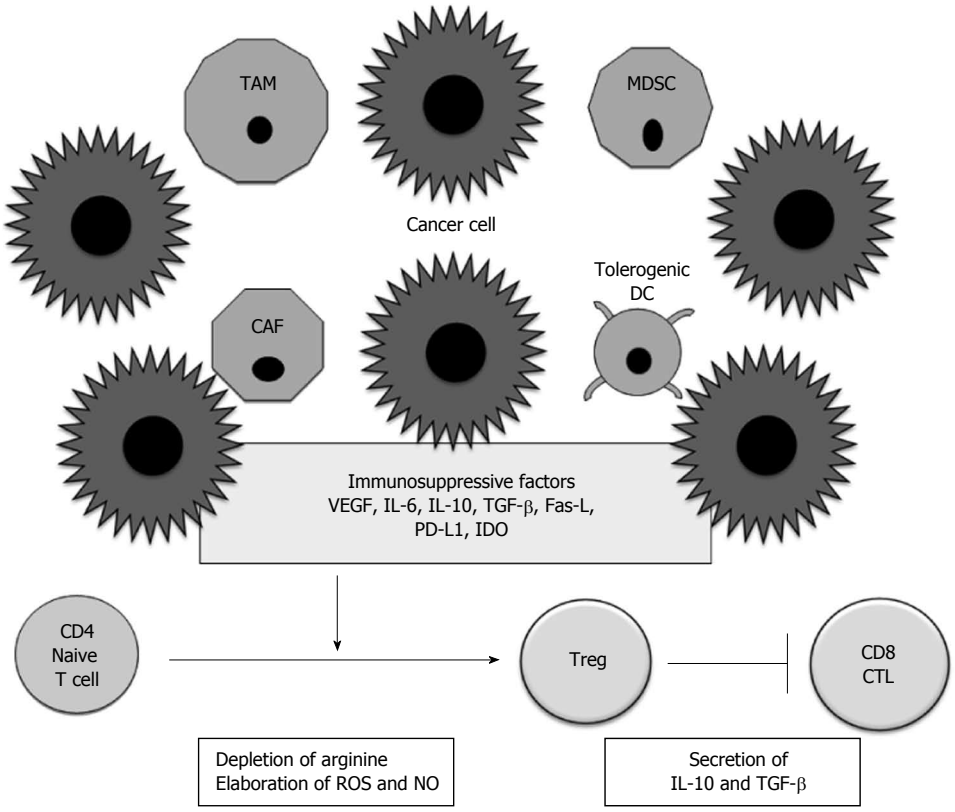

Figure 1 Immunosuppression in the tumor microenvironment. Cancer cells secrete various factors such as vascular endothelial growth factor (VEGF), interleukin (IL)-6, IL-10, transforming growth factor-β (TGF-β), Fas ligand (FasL), PD1 ligand 1 (PD-L1), and indolamine-2,3-dioxygenase (IDO), all of which promote the accumulation of heterogeneous populations of cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs), and tolerogenic dendritic cells (DCs). These immunosuppressive cells inhibit antitumor immunity by various mechanisms, including depletion of arginine and elaboration of reactive oxygen species (ROS) and nitric oxide (NO). The tumor microenvironment promotes the accumulation of Tregs that suppress CD8+ cytotoxic T lymphocyte (CTL) function through secretion of IL-10 and TGF-β.

mutant ras peptide, 2 of 7 CRC patients showed antitumor immune responses that were significantly associated with prolonged overall survival[53]. Moreover, in a phase Ⅱ trial, vaccination with the β-hCG peptide induced anti-β-hCG antibody production in 56 of 77 CRC patients[46]. Interestingly, anti-β-hCG antibody induction was associated with longer overall survival[46]. However, some clinical trials report a discrepancy between clinical and immunological responses. In SART3 peptide vaccine therapy, IgE-type anti-peptide antibodies were detected after vaccination; however, immunological responses were not detected in the patients[45]. Peptide vaccines for CRC patients are generally well-tolerated, with no patients requiring cessation due to toxicity; however, a high frequency of reactions were observed at the injection site due to the use of adjuvants such as incomplete Freund's adjuvant, IL-2, granulocyte-macrophage colony-stimulating factor (GM-CSF), and bacillus Calmette-Guerin (BCG). Importantly, peptide vaccines have shown only limited success in clinical trials. There are several drawbacks to the peptide vaccination strategy, including: (1) limitations due to the patient's HLA type[54]; (2) ineffectiveness of CD8+ CTLs due to the down-regulation of certain antigens and MHC class I molecules; (3) impaired DC function in patients with advanced cancer[55]; and (4) tumor microenvironments, where immune suppressive cells such as Tregs exist[13]. Synthetic long peptides may be more attractive candidates for peptide-based vaccines. In a phase I/Ⅱ trial, 10 CRC patients were vaccinated twice with a set of 10 overlapping p53 synthetic long peptides[50]. P53-specific CD4+ and CD8+ T-cell responses were observed in 9 of 10 CRC patients, and 6 of 9 tested patients maintained p53-specific T-cell reactivity for at least 6 mo. New trials may focus on improving the long peptide vaccine-induced antitumor immune responses.

DC vaccines

Three signals were required for induction of efficient CTL responses: (1) simultaneous presentation of multiple TAAs by both MHC class I and class Ⅱ molecules; (2) costimulation by membrane-bound receptor-ligand pairs; and (3) cytokines to direct polarization of the resultant antitumor immune responses. DCs can provide all three of these signals that are essential for the induction of antitumor immunity[33]. Therefore, many clinical trials of antigen-pulsed DCs have been conducted in patients with various types of tumors, including CRC.

To date, various strategies for delivering TAAs to DCs have been developed to generate potent CTL responses against tumor cells. DCs have been pulsed with synthetic peptides derived from the known TAAs[56], tumor cell lysates[57], apoptotic tumor cells[32], and tumor RNA[58], or

Table 1 Immunotherapy strategies for colorectal cancer

Vaccine	Clinical response	Immunological response	Ref.
Peptide			
Survivin-2B	PR (1/15) SD (3/15) PD (11/15) Temporary decrease of CEA level 40% (6/15)	Increase of Survivin-2B-specific CTL frequency DTH 40% (6/15)	[48]
Combination chemotherapy with peptide vaccine against RNF43 and TOMM34	SD (16/19) PD (3/19)	8 of 21 patients exhibited antigen-specific CTL responses to both RNF43 and TOMM34, and 12 patients exhibited CTL responses to one of the peptides only	[52]
13-mer mutant ras	Of nine patients who completed all six vaccinations, seven patients showed no remaining evidence of disease	Two CRC patients showed immunological responses, and the antitumor immune response was significantly associated with prolonged overall survival	[53]
β-hCG	Prolongation of survival in patients with a high level of antipeptide antibodies	Induction of serum antipeptide antibody (56/77)	[46]
SART3	Diagnosis at 5 mo after first vaccination: SD (1/19) PD (10/19)	Increased CTL activity (2/11), induction of serum antipeptide IgG (2/12), IgE (5/12), DTH (0/12)	[45]
A set of 10 overlapping p53 synthetic long peptides		Induction of p53-specific CD4+ and CD8+ T-cell responses (9/10), maintained p53-specific CTL reactivity for at least 6 mo (6/9)	[50]
DC			
DC pulsed with CEA peptide or CEA mRNA	Disease stabilization was observed in several patients	The majority of CRC patients demonstrated induction of CEA-specific T cell responses	[60-65]
DCs pulsed with CEA-derived altered peptides combined with the adjuvant Flt3 ligand	2 of 12 patients exhibited SD for 3 and 6 mo; 2 patients exhibited CR for more than 10 mo; 1 patient had a mixed response with some regression of liver metastases	Expansion of CD8+ T cells that recognize both the native and altered epitopes and possess an effector CTL phenotype	[64]
Whole tumor cell			
Autologous tumor cells combined with BCG	No significant clinical benefit was seen with whole tumor cell vaccines in surgically resected patients with stage Ⅱ or Ⅲ CRC	When treatment compliance was evaluated, the trend indicated benefits from vaccination in terms of disease-free survival (P = 0.078) and overall survival (*P* = 0.12)	[68]
NDV-infected irradiated autologous tumor cells	A randomized phase Ⅲ study of 50 patients with resectable CRC liver metastases demonstrated that vaccination with NDV-infected whole tumor cell did not significantly improve overall survival.	DTH (21/31)	[74,75]
Viral vector			
Replication-defective recombinant fowlpox and vaccinia viruses encoding the CEA antigen and TRICOM (B7.1, ICAM-1, and LFA-3)	SD (3/9)	Induction of CEA-specific CTL (3/9)	[79]
Combination chemotherapy and vaccination with a nonreplicating canarypox virus (ALVAC) expressing the CEA and T-cell costimulatory molecule B7.1 (ALVAC-CEA/B7.1)	Objective response (42/118)	Increases in CEA-specific T cells were detected in patients treated with chemotherapy and booster vaccination	[80]

Immunotherapy strategies including peptides derived from tumor-associated antigens, whole tumor cells, *in vitro*-generated dendritic cells (DCs), or viral vector-based cancer vaccine. PD: Programmed cell death protein; CTL: Cytotoxic T lymphocytes; CEA: Carcinoembryonic antigen; CRC: Colorectal cancer; RNF43: Ring finger protein 43; TOMM34: Translocase of outer mitochondrial membrane 34; β-hCG: β-human chorionic gonadotropin; SART3: Squamous cell carcinoma antigen recognized by T cells 3; NDV: Newcastle disease virus.

physically fused with whole tumor cells[59] to induce efficient antitumor immune responses (Figure 2). Because CEA is a tumor-associated antigen expressed by most CRCs, DCs have been pulsed with CEA peptides[60-64] or CEA mRNA[63,65]. In these phase I clinical trials, the majority of vaccinated CRC patients demonstrated the induction of CEA-specific T cell responses. Moreover, disease progression stabilized in several patients, and the vaccine was safe and well-tolerated. As CEA is a self-antigen and poorly immunogenic, Fong *et al*[64] generated altered peptide ligands that were derived from native T cell epitopes and contained amino acid substitutions that either increased the peptide affinity for the MHC peptide-binding groove or modified interactions with the T cell receptor. In this trial, 12 patients were immunized with DCs loaded with altered peptides derived from CEA and the Flt3 adjuvant ligand. Two of 12 patients showed disease stabilization for 3 mo and 6 mo, 2 patients showed complete responses for more than 10 mo, and one patient had a mixed response with some regression of liver metastases. To improve the clinical efficacy of DC-based cancer vaccines, it is crucial to design novel strategies that boost

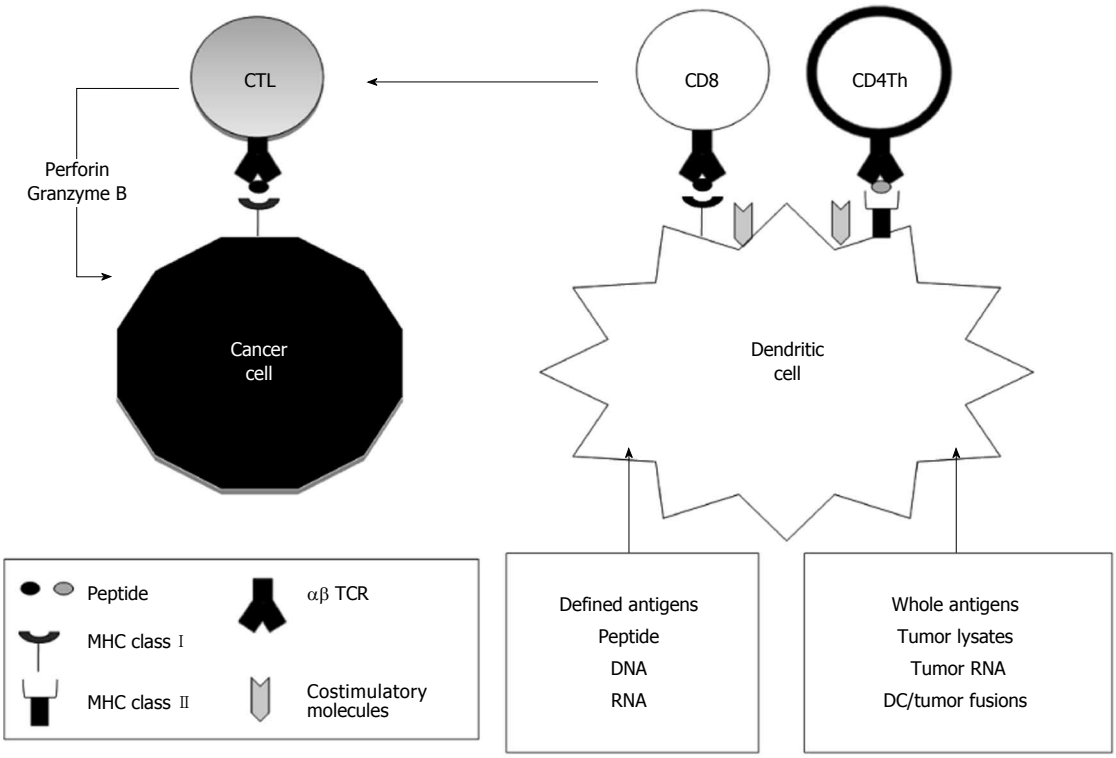

Figure 2 Dendritic cell vaccines. Dendritic cells (DCs) are loaded with antigens, which are taken up and degraded into peptide fragments by antigen-presenting cells such as immature DCs. DCs process tumor-derived peptides and major histocompatibility complex (MHC) class Ⅰ peptides derived from DCs. They form MHC class Ⅰ-peptide complexes in the endoplasmic reticulum, which are then transported to the surface of the DCs and presented to CD8+ T cells. DCs also synthesize MHC class Ⅱ peptides in the endoplasmic reticulum, which are transported to the cytoplasm where MHC class Ⅱ-peptide complexes are assembled with tumor-derived peptides and presented to CD4+ T cells. CD8+ T cells become cytotoxic T lymphocytes (CTLs), which destroy cancer cells through effector molecules such as perforin and granzyme B. TCR: T cell receptor.

adaptive antitumor immunity to overcome tolerance.

Whole tumor cell vaccines

Because autologous tumor cells express a whole TAAs including those known and unidentified, using whole tumor cells could greatly diminish the chance of tumor escape compared to using a single epitope peptide[41,42]. A significant disadvantage to this approach is the difficulty in generating a "universal" vaccine that could be applicable to all patients with a given cancer. Autologous whole tumor cells have been used as cancer vaccines to induce polyclonal CTL induction in several cancer types[66,67], including CRC[68]. A randomized phase Ⅲ clinical trial of a combined autologous whole tumor cell plus BCG vaccine was conducted to determine whether surgical resection plus vaccination was more beneficial than resection alone in 412 stage Ⅱ and Ⅲ CRC patients. This study showed no significant clinical benefit from whole tumor cell vaccination in surgically resected patients with stage Ⅱ or Ⅲ CRC. However, effective immune responses were associated with the improved disease-free and overall survival. Only a small proportion of the proteins in an autologous whole tumor cell vaccine are specific to tumor cells, while a vast majority of antigens in the vaccine are shared with normal cells. Moreover, whole tumor cell vaccines are likely to be poorly immunogenic. Therefore, the immune response generated by whole tumor cell vaccines is generally insufficient to provide benefit to patients. To improve the immunogenicity of whole tumor cell vaccines, autologous tumor cells have been genetically modified to secrete GM-CSF and then re-administered to the patient[69]. The trials have shown promising results in patients with prostate carcinoma[70], renal cell carcinoma[71], metastatic non-small-cell lung carcinoma[72], and melanoma[73]. This approach is based on the fact that GM-CSF is required at the site of the tumor to effectively prime TAA-specific immunity[69]. Another tumor cell vaccine approach utilizes Newcastle disease virus (NDV)-infected irradiated tumor cells as autologous CRC vaccines[74]. This approach resulted in a 97.9% two-year survival rate in patients with resected CRC, compared to 66.7% when treated with autologous tumor cells combined with BCG. However, a randomized phase Ⅲ study of 50 patients with resectable CRC liver metastases demonstrated that vaccination with NDV-infected whole tumor cells did not significantly improve overall survival, disease-free survival or metastases-free survival, although subgroup analyses suggested that the vaccines were somewhat beneficial[75]. The immunogenicity of whole tumor cells needs to be improved for this vaccination strategy to be effective. However, it is unclear which specific agents (such as cytotoxic chemotherapeutics, ionizing irradiation, and chemical agents) are best suited for killing tumor cells to generate highly immunogenic whole tumor cell vaccines.

Viral vector vaccines

Recombinant viral vectors are potentially useful vaccine vehicles for cancer therapy. Many types of recombinant viruses are naturally immunogenic, infect APCs (specifically DCs), and express TAAs[76]. The natural immunogenicity of viral vectors acts as an adjuvant to help boost TAA-specific immune responses. In one study, CRC patients were immunized with vaccinia virus or a replication-defective avian poxvirus encoding CEA. In this phase I study, viral-based vaccination with replication-defective recombinant fowlpox and vaccinia viruses encoding the CEA antigen and TRICOM (B7.1, ICAM-1, and LFA-3) induced CEA-specific T cell responses[77] and disease stabilization in 40% of patients with metastatic cancer (including CRC) for at least 4 mo[78]. A phase Ⅱ clinical trial in patients with metastatic CRC examined the efficacy of chemotherapy combined with vaccination with a nonreplicating canarypox virus (ALVAC) expressing the CEA and T-cell costimulatory molecule, B7.1 (ALVAC-CEA/B7.1). Anti-CEA-specific T cell responses were successfully generated in 50% of patients undergoing chemotherapy and booster vaccination. Objective clinical responses were observed in 40% of the patients[79,80]. Interestingly, chemotherapy does not appear to inhibit vaccine-mediated immunity.

ADOPTIVE CELL TRANSFER THERAPY

Cancer immunotherapy can be either active or passive. In passive cellular immunotherapy, specific effector cells are directly infused and are not induced or expanded within the cancer patient. Because most tumor cells express MHC class I -peptide, which can be recognized by antigen-specific CD8+ CTLs. Therefore, adoptive transfer of activated CTLs successfully used in patients with advanced cancer[81]. In adoptive cell transfer therapy (ACT), autologous T cells are removed from patients, activated, expanded to large numbers *in vitro* and transferred back into the patients. ACT overcomes tolerogenic mechanisms by enabling the selection and activation of highly reactive T cell subpopulations and manipulating the host environment into which the T cells are introduced. Indeed, upon the successful induction of specific CTLs *in vitro*, a clinical response to adoptive immunotherapy can be expected even in patients with advanced CRC[82]. Moreover, injection of IFN promotes the MHC class I -peptide on the cell surface, thus antitumor immune responses are augmented. However, there are several drawbacks to ACT that should be considered, including a potential lack of immune memory, poor persistence of activated effector cells in patients, the prohibitive expense, and the time required to expand the cells.

A new approach using T cells genetically modified to express receptors that recognize TAAs with high specificity to tumor cells may provide significant clinical benefits, especially for large solid tumors[83]. Recently, several clinical trials have demonstrated the therapeutic potential of this approach, which lead to impressive tumor regression in cancer patients[84]. A phase I trial in CRC patients examined human T cells engineered to express a high-avidity CEA-specific TCRs[85]. In this study, autologous T cells genetically engineered to express a murine TCR against human CEA were administered to three patients with metastatic colorectal cancer that was refractory to standard treatments. All patients experienced profound decreases in serum CEA levels (74%-99%), and one patient had an objective regression of cancer metastatic to the lung and liver. However, all three patients developed severe transient inflammatory colitis.

ANTIBODY-BASED CANCER IMMUNOTHERAPY

Monoclonal antibodies (mAbs) that target surface antigens expressed on tumor cells are clinically effective as cancer therapeutics[86]. Three mAbs (Cetuximab, Bevacizumab and Panitumumab) are approved for the treatment of CRC in the United States, and many other mAbs are being tested in clinical trials[87]. Treatment with mAbs can induce tumor cell death by several mechanisms, including interference with vital signaling pathways. Moreover, it is becoming apparent that innate immune effector mechanisms that engage the Fc portion of the antibody *via* Fc receptors are equally important[88]. The immune cytotoxicity includes ADCC, complement-mediated cytotoxicity, and antibody-dependent cellular phagocytosis. Bevacizumab, a recombinant humanized monoclonal antibody that selectively binds to human VEGF, is effective in KRAS wild-type CRC patients[89]. Recent evidence has also shown clinical benefits from treatment with anti-EGFR, Cetuximab and Panitumumab in KRAS wild-type CRC patients[90].

CYTOKINE THERAPY

Cytokines are substances proteins and glycoproteins that are secreted by immune cells. They have autocrine and paracrine functions and function locally or at a distance to enhance or suppress antitumor immunity. To date, IL-2 and IFN-α are two cytokines approved by the FDA for cancer therapy. Cytokines may be useful for treating hematologic malignancies (hairy cell leukemia and chronic myelogenous leukemia) or immunogenic tumors (melanoma and renal cell carcinoma). The major cytokines currently in use or under evaluation for cancer therapy are IFN-α, IL-2, GM-CSF, and IL-12.

COMBINED IMMUNOTHERAPY

It is well known that even if CRC appears to have been eradicated by chemotherapy and radiation, a small cancer stem cell (CSC) fraction that can self-propagate and sustain tumor growth frequently persists, leading to relapse and therapeutic failure. Although CSC is often resistant to a variety of treatments, including chemotherapy and radiotherapy, immunotherapy may still be effective[8-10]. A combined approach that uses conventional chemotherapy

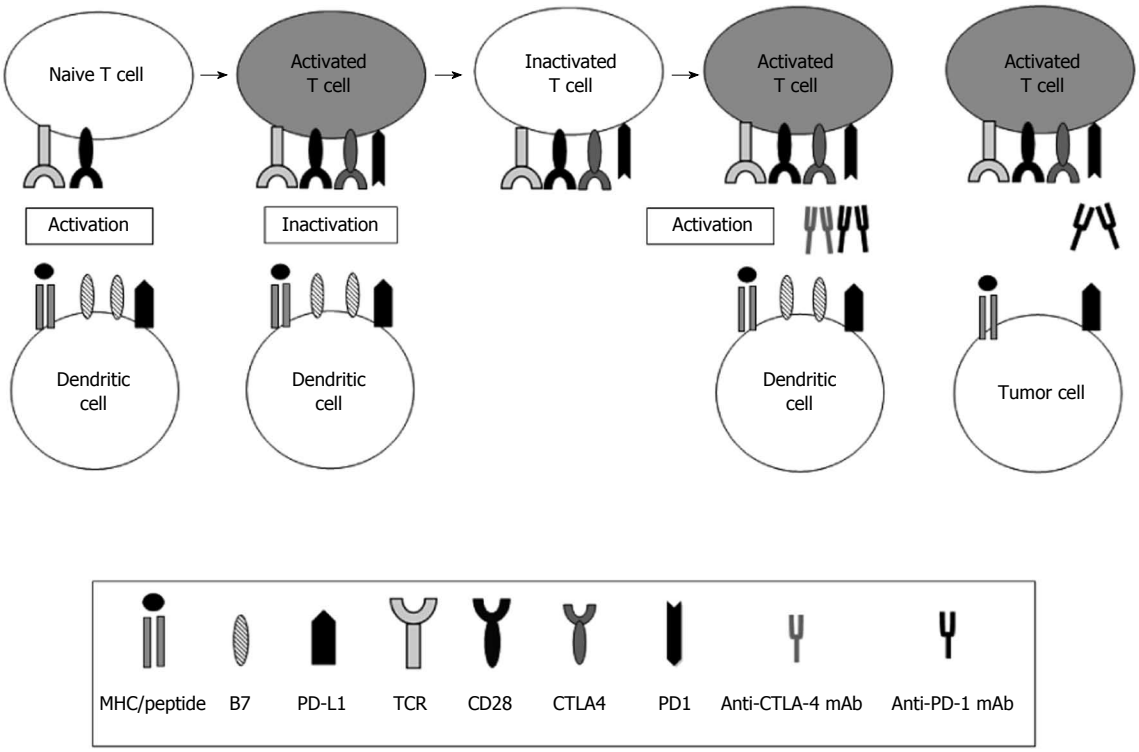

Figure 3 Immune regulatory checkpoints in cancer immunotherapy. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death protein 1 (PD1) are two well-described co-inhibitory molecules that are expressed on naïve or memory T cells and decrease antitumor immune responses. The CTLA-4-mediated immune checkpoint is induced in T cells at the initial response to antigen (early activation phase). After the T cell receptor (TCR) is triggered by antigen encounter, CTLA-4 is transported to the surface of naïve or memory T cells. In contrast, the major role of the PD1 pathway is not at the initial T cell activation stage but rather the regulation of inflammatory responses by effector T cells that recognize antigen in peripheral tissue cells. Thus, PD-1 is highly expressed by antigen-specific cytotoxic T lymphocytes (CTLs) in malignancies and is associated with impaired T-cell function. The best-characterized signal for PD1 ligand 1 (PD-L1) induction is interferon-γ (IFN-γ), which is predominantly produced by Th1 cells. Although PD-L2 expression is limited to dendritic cells (DCs) and macrophages, PD-L1 is broadly expressed in tissues and is considered a molecular shield that protects cells from auto-reactive attack. In some tumors, PDL1 is not constitutively expressed but is induced in response to inflammatory signals that are produced by an active antitumor immune response. Loading DCs with soluble PD1 decreases their function. Therefore, antibodies can be used to block inhibitory ligand:receptor interactions by acting on tumor cells, DCs (*e.g.*, anti-PD-L1) or T cells (*e.g.*, anti-CTLA-4 or anti-PD1). Combining the blockade of multiple inhibitory pathways synergistically decreases T cell anergy and improves T cell responsiveness against tumors.

or radiation to kill the bulk of cancer cells and immunotherapy to keep residual CSCs and differentiated cancer cells in check may abrogate the replenishing pool of CRC cells[91]. In addition, treatment with chemotherapy such as cyclophosphamide or gemcitabine can augment the antitumor effects of cancer immunotherapy by depleting Treg, potentially enhancing antitumor immune responses[92]. Therefore, chemotherapy can kill cancer cells and boost antitumor immune responses all at the same time[93,94]. A recent study reported that immune checkpoint blockade with monoclonal antibodies targeting the inhibitory immune receptors cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), PD-1, and PD-L1 can be used to successfully treat patients with advanced melanoma (Figure 3)[95-98]. Combined, these approaches have the potential to significantly improve patient outcomes compared to treatment with conventional cancer therapies such as chemotherapy, radiation, monoclonal antibodies, hormonal therapy, and photodynamic therapy.

FUTURE PERSPECTIVE

Improved treatment options that selectively target cancer-dependent pathways with little or no toxicity to normal tissues are urgently needed. Work in our laboratory focuses on these key aspects by combining the use of DCs pulsed with MHC class I and II peptides and conventional chemotherapy. Immunotherapy may be combined with conventional therapy to reduce Tregs and enhance CTL responses. Knockdown of PD-L1 and PD-L2 on monocyte-derived DCs and tumor cells may help decrease tolerance (Figure 3). DCs electroporated with PD-L small-interfering RNAs are highly effective in enhancing T cell proliferation and cytokine production and are therefore attractive candidates for improving the efficacy of DC vaccines in cancer patients[99]. Moreover, combined blockade of PD1 and CTLA-4, which play key roles in inhibiting T-cell activation, enhances antitumor immune responses compared to either agent alone (Figure 3)[100]. Combining immunotherapies, particularly agents that target different immune checkpoints, may be a promising approach. Preliminary clinical findings indicate that combined targeted therapies and simultaneous blockade of multiple immune checkpoints could promote therapeutic synergy and long-term antitumor immunity to improve clinical outcomes for melanoma patients[101]. In the metastatic CT26 CRC mouse model, simultaneous blockade of CTLA-4 and PD-L1 enhanced antitumor

activity in an interleukin-15-dependent manner[102].

CONCLUSION

The limitations of surgery and adjuvant chemo/radio/antibody therapies in treating CRC patients necessitate the development of novel approaches, including immunotherapy. Despite tremendous progress in basic immunological research, effective immunotherapies for most types of cancer, including CRC, are still lacking. Immunotherapy alone may be insufficient for treating advanced CRC patients. The most promising therapeutic approach for activating antitumor immunity in cancer patients may be blockade of inhibitory immune regulatory proteins such as immune checkpoint ligands and receptors. Therefore, it is important to develop cancer vaccines that do not express inhibitory molecules such as PD-L1, but do express high levels of molecules that enhance CTL priming, such as CD80 and 4-1BBL. The blockade of multiple immune regulatory checkpoints combined with immunotherapy and/or conventional therapy may be effective in treating patients with advanced CRC.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Early rehabilitation programs after laparoscopic colorectal surgery: Evidence and criticism

Duck-Woo Kim, Sung-Bum Kang, Soo-Young Lee, Heung-Kwon Oh, Myung-Hoon In

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Abstract

During the past several decades, early rehabilitation programs for the care of patients with colorectal surgery have gained popularity. Several randomized controlled trials and meta-analyses have confirmed that the implementation of these evidence-based detailed perioperative care protocols is useful for early recovery of patients after colorectal resection. Patients cared for based on these protocols had a rapid recovery of bowel movement, shortened length of hospital stay, and fewer complications compared with traditional care programs. However, most of the previous evidence was obtained from studies of early rehabilitation programs adapted to open colonic resection. Currently, limited evidence exists on the effects of early rehabilitation after laparoscopic rectal resection, although this procedure seems to be associated with a higher morbidity than that reported with traditional care. In this article, we review previous studies and guidelines on early rehabilitation programs in patients undergoing rectal surgery. We investigated the status of early rehabilitation programs in rectal surgery and analyzed the limitations of these studies. We also summarized indications and detailed protocol components of current early rehabilitation programs after rectal surgery, focusing on laparoscopic resection.

Key words: Colorectal cancer; Enhanced recovery after surgery; Early rehabilitation; Fast-track; Laparoscopy; Rectal

Core tip: Several randomized trials and meta-analyses have confirmed that the implementation of early rehabilitation programs for perioperative care is useful for recovery of patients after colorectal resection. However, most of the previous evidence is obtained from studies of early rehabilitation programs adapted to open colonic resection. Currently, early rehabilitation combined with laparoscopic rectal surgery can be a feasible alternative in some selected patients, but indications are not established. Current evidence fails to support the safety of early rehabilitation combined with laparoscopic rectal surgery compared to that reported for laparoscopic colon surgery.

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INTRODUCTION

Previously, patients undergoing colorectal surgery received traditional perioperative care, which comprised sufficient mechanical bowel preparation, insertion of a

nasogastric tube, preoperative fasting, postoperative fasting for up to 1 wk, and multiple intra-abdominal drains. Eventually, early rehabilitation programs were developed to decrease postoperative pain, perioperative physiological stress, and organ dysfunction, and to promote patient motivation, leading to enhanced recovery after surgery; decreased postoperative morbidity, length of hospital stay, and health care resources; and improved overall outcomes[1]. Since their first introduction in the mid-1990s, early rehabilitation programs, also known as fast-track pathways or enhanced recovery after surgery (ERAS), have become increasingly popular in the care for patients with colorectal surgery[2].

During the past several decades, many studies have reported the results of early rehabilitation programs in colorectal surgery. Several randomized controlled trials and meta-analyses have indicated that the implementation of these evidence-based detailed perioperative care protocols is useful for early recovery of patients after colorectal resection[3-7]. Patients who underwent these programs showed rapid recovery of bowel movement, shortened length of hospital stay, and fewer complications compared with traditional care programs. However, most evidence from previous studies corresponded to patients undergoing colonic surgery for various diseases. Currently, the strongest evidence for early rehabilitation programs was adopted from open colonic resection[8]. At present, early rehabilitation programs in rectal surgery require standardization and can be adopted only after validation with high-level evidence from well-designed randomized controlled trials.

In this review, we summarized early rehabilitation programs reported in previous studies and guidelines including patients undergoing rectal surgery, and we analyzed the limitations of these studies. We also summarized indications and details of current early rehabilitation programs after rectal surgery, focusing on a laparoscopic resection perspective.

EARLY REHABILITATION PROGRAMS AFTER RECTAL SURGERY: STATUS QUO

Early rehabilitation and laparoscopic colonic surgery

Laparoscopic colorectal surgery has been established as a comparable alternative to open surgery with respect to its feasibility, safety and long-term outcomes. For malignant diseases, laparoscopic colonic resection performed by an experienced surgeon involves adequate lymph node harvest, sufficient surgical margins, and reduced operative time and intraoperative blood loss[8]. A previous study suggested that laparoscopic surgery could reduce the prevalence of postoperative immunosuppression[9]. Prospective randomized trials have shown that laparoscopic surgery for colon cancer can achieve earlier recovery in organ function and long-term oncological results equal to those for open colonic resection[10-12]. However, these trials did not apply early rehabilitation programs. Both laparoscopic surgery and early rehabilitation programs focus on minimizing surgical pain and perioperative stress, and enhancing recovery after surgery. Many cohort series, meta-analyses, and several prospective randomized studies showed early rehabilitation programs and laparoscopic surgery can have a synergistic effect in enhancing recovery after laparoscopic surgery for colon disease[9,13,14]. Recently, the Laparoscopy and/or Fast-track Multimodal Management Versus Standard Care (LAFA) study, the largest multicenter randomized controlled trial thus far, reported comparative results between laparoscopic and open colectomy[9]. The total length of hospital stay was 2 d less than that after laparoscopic surgery. Furthermore, laparoscopic surgery was the only predictive factor associated with reduced hospital stay and morbidity. The results from the LAFA study also indicated that early oral intake, early mobilization, and laparoscopic surgery were independent determinants of early recovery[9,15]. In a previous study, we evaluated the efficacy of a rehabilitation program after laparoscopic colon surgery in the context of a randomized controlled trial. We found that the recovery time was shorter in the early rehabilitation program group than in the conventional care group, without differences in complication rates, quality of life, and pain[13]. Previous studies representative of laparoscopic colon surgery with early rehabilitation are summarized in Table 1. As early rehabilitation programs became more popular in the management of patients undergoing colon surgery, an international collaborative research group proposed a set of guidelines for perioperative care in elective colonic surgery, with the participation of the ERAS Society for Perioperative Care, The European Society for Clinical Nutrition and Metabolism, and The International Association for Surgical Metabolism and Nutrition[16]. These guidelines recommend detailed protocols for each component ranging from patient selection to hospital discharge, and provide additional consideration points in the setting of laparoscopic surgery.

Early rehabilitation and laparoscopic rectal surgery

Laparoscopic rectal resection for various benign and malignant diseases, including total mesorectal excision, is considered technically challenging and has not gained popularity compared to laparoscopic colon resection. However, several studies have demonstrated that it is a feasible and safe alternative to open rectal surgery; some authors have reported that the short- and long-term oncological results were equal to those with open surgery[17-20]. We also reported the results of our multicenter study comparing open *vs* laparoscopic surgery for mid-rectal and low rectal cancer after neoadjuvant chemoradiotherapy (COREAN trial), which showed that laparoscopic surgery was safe and had short-term benefits, including earlier recovery of bowel function, shorter time to resume a normal diet, shorter time to first defecation, and less requirement for morphine, compared with open surgery[21]. Similarly, the quality of oncological resection was equivalent. Patients enrolled in the COREAN trial received postoperative management consisting of tradi-

Table 1 Previous representative studies of colonic surgery with early rehabilitation programs

Ref.	Country	Study design	Inclusion period	Patients (*n*)	Operations	Approach	LOS (d)		Readmissions		Morbidity		Mortality	
							ERP	CC	ERP	CC	ERP	CC	ERP	CC
Anderson *et al*[3], 2003	United Kingdom	RCT	ND	25 (ERP: 14, CC: 11) Cancer: 18 (72) ERP: 11, CC: 7	RH: 14 (ERP: 9, CC: 5) LH: 11 (ERP: 5, CC: 6)	ND	3 (2-7)	7 (4-10)[a]	0 (0)	0 (0)	4 (29)	5 (45)	0 (0)	1 (9)
Gatt *et al*[4], 2005	United Kingdom	RCT	ND	39 (ERP: 19, CC: 20) Cancer: 27 (69) ERP: 12, CC: 15	RH: 10 (ERP: 5, CC: 5) AR: 15 (ERP: 5, CC: 10) Others: 14 (ERP: 9, CC: 5)	ND	5 (4-9)	7.5 (6-10)[a]	1 (5)	4 (20)	9 (47)	15 (75)	1 (5)	0 (0)
Khoo *et al*[5], 2007	United Kingdom	RCT	2003-2004	70 (ERP: 35, CC: 35) Cancer: 70 (100)	Colonic: 47 (ERP: 22, CC: 25) Rectal: 23 (ERP: 13, CC: 10)	Open	5 (3-37)	7 (4-63)[a]	3 (9)	1 (3)	9 (26)	16 (46)	0 (0)	2 (6)
Muller *et al*[6], 2009	Switzerland	RCT	2004-2006	151 (ERP: 76, CC: 75) Cancer: 131 (87) ERP: 67, CC: 64	RH: 48 (ERP: 26, CC: 22) AR/LH: 101 (ERP: 30, CC: 51)	Open	5 (2-30)	9 (6-30)[a]	3 (4)	2 (3)	16 (21)	37 (49)[a]	0 (0)	0 (0)
Serclova *et al*[7], 2009	Czech	RCT	2005-2007	103 (ERP: 51, CC: 52) Cancer: 7 (7) ERP: 3, CC: 4 IBD: 89 (86) ERP: 46, CC: 43	Simple: (ERP: 47.1%, CC: 61.5) Multiple: (ERP: 29.4%, CC: 21.2)	Open	7 (5-11)	9 (7-22)[a]	0 (0)	0 (0)	11 (22)	25 (48)[a]	0 (0)	0 (0)
Lee *et al*[13], 2011	South Korea	RCT	2007-2009	100 (ERP: 46, CC: 54) Cancer: 100 (100)	RH: 38 (ERP: 17, CC: 21) LH: 15 (ERP: 5, CC: 10) AR: 47 (ERP: 24, CC: 23)	Lap	7 (6-8)	8 (7-9)	0 (0)	0 (0)	6 (11)	14 (20)	0 (0)	0 (0)
Vlug *et al*[9], 2011	Netherlands	RCT	200 -2009	400 (ERP: 193, CC: 207) Cancer: 400 (100)	RH: 179 (ERP: 80, CC: 99) LH: 221 (ERP: 120, CC: 101)	Open/lap	Open: 7 (5-11) Lap: 5 (4-8)	Open: 7 (6-13) Lap: 6 (4.5-9.5)[a]	13 (7)	14 (7)	125 (65)	132 (64)	6 (3)	4 (2)
Wang *et al*[26], 2012	China	RCT	2006-2009	78 (ERP: 40, CC: 38) Cancer: 78 (100)	RH: 13 (ERP: 7, CC: 6) Sig: 34 (ERP: 18, CC:16) AR: 25 (ERP: 13, CC: 12)	Lap	5.5 (5-6)	7.0 (6-8)[a]	ND	ND	2 (5)	8 (21)	0 (0)	0 (0)

[a]$P < 0.05$ *vs* early rehabilitation program (ERP) group. LOS: Length of hospital stay; CC: Conventional care; RCT: Randomized controlled trial; RH: Right hemicolectomy; LH: Left hemicolectomy; SB: Small bowel; AR: Anterior resection; IBD: Inflammatory bowel disease; Lap: Laparoscopic; LAR: Low anterior resection; APR: Abdominoperineal resection; Sig: Sigmoidectomy; ND: Not documented. Continuous data are given as median (range) or mean ± SD.

tional standard care instead of an early rehabilitation program. Only a few study results support the hypothesis that laparoscopic rectal surgery and a subsequent early rehabilitation program can act synergistically to enhance postoperative recovery and surgical outcomes.

During the past decade, some studies including prospective cohort studies and randomized controlled trials have shown that early rehabilitation programs enhance recovery after laparoscopic rectal resection and shorten the length of hospital stay[22-26]. However, these studies were heterogeneous: mixed open surgery or laparoscopy, colorectal disease or rectal disease, diverting stoma, and sphincter preservation, which makes it difficult to accept the validity of their results. Additionally, differences exist in the detailed components of individual early rehabilitation programs, which are classified into three categories of preoperative preparation, intraoperative intervention, and postoperative management, making it difficult to interpret a causal relationship between the components and positive/negative outcomes. To the best of our knowledge and based on the results of this literature review, only five studies have reported the results of implementation of early rehabilitation programs after laparoscopic rectal surgery: three prospective cohort studies[22,27,28], one retrospective case-control study[29] and one randomized controlled trial[30]. The characteristics of these studies are summarized in Table 2.

A prospective cohort study by Lindsetmo *et al*[22] reported the results of 37 patients undergoing laparoscopic rectal resection. The mean hospital stay was 3.0 d (range, 1-8 d), in which 90% of patients were discharged < 5 d after surgery. No anastomotic leaks or mortality occurred, and the in-hospital complication rate was 8% (1 surgical-site infection

Table 2 Summary of previous studies that evaluated early rehabilitation programs after laparoscopic rectal surgery

Ref.	Country	Study design	Inclusion period	Patients (*n*)	Operations	Clinical effectiveness (LOS and complications)
Lindsetmo *et al*[22], 2009	United States	Prospective cohort study	2005-2007	37 Cancer: 17 (46) Polyp: 4 (11) Others: 16 (43)	SPS: 37 (100) Diverting ileostomy: 7 (19)	Mean LOS: 3.0 d (range 1-8 d) Overall complications: 6 (16) UTI: 1; SSI: 2 Readmission < 30 d: 3 (8)
Chen *et al*[27], 2011	Taiwan	Prospective cohort study	2007-2009	80 Cancer: 76 (95) Benign: 4 (5)	APR: 15 (19) SPS: 65 (81) Diverting ileostomy: 32 (49)	Mean LOS: 5.0d (range 3-22) Overall complications: 11 (14) AL: 1; pelvic abscess 2; ileus: 1 Readmission < 30 d: 7 (9)
Stottmeier *et al*[28], 2012	Denmark	Prospective cohort study	2006-2009	102 Cancer: 102 (100)	APR: 19 (19) Hartmann: 6 (6) SPS: 77 (75) Diverting colostomy: 38 (37) Diverting ileostomy: 3 (3)	Median LOS: 5 d (range 2-42 d) Overall complications: 25 (25) AL: 3; intra-abdominal abscess: 3 Readmission < 30 d: 15 (15)
Huibers *et al*[29], 2012	Nether-lands	Retrospective case-control study	2004-2009	76 (ERP: 43, CC: 33) Cancer: 76 (100)	APR: 24 (32) ERP: 16 (37) CC: 8 (24) SPS: 52 (68) ERP: 27 (63) CC: 25 (76)	Median LOS: (P = 0.042) ERP: 7 d (range 2-83 d) CC: 10 d (range 4-74 d) Overall complications: ERP: 17 (40) AL: 5; intra-abdominal abscess: 7 CC: 9 (27) AL: 4; intra-abdominal abscess: 3 Readmission < 30 d: (P = 0.421) ERP: 5 (12) CC: 6 (18)
Lee *et al*[30], 2013	South Korea	RCT	2007-2011	98 (ERP: 52, CC: 46) Cancer 98 (100)	SPS: 98 (100) Diverting ileostomy: 98 (100)	Median recovery time[1]: (P = 0.47) ERP: 137 h (range 107-188 h) CC: 146.5 h (range 115-183 h) Overall complications: (P = 0.054) ERP: 22 (42) AL: 1; POI: 15; acute voiding difficulty: 9 CC: 11 (24) AL: 1; POI: 6; acute voiding difficulty: 2 Readmission < 30 d: 0 (0)

[1]Defined by tolerable diet for 24 h, safe ambulation, analgesic-free and afebrile without complication. LOS: Length of hospital stay; SPS: Sphincter preserving surgery; UTI: Urinary tract infection; SSI: Surgical site infection; APR: Abdominoperineal resection; AL: anastomosis leakage; ERP: Early rehabilitation program; CC: Conventional care; RCT: Randomized controlled trial; POI: Postoperative ileus.

and 1 urinary tract infection). Readmission was required in three patients (8%) because of medical illness. The authors suggested that laparoscopy in conjunction with modern perioperative care allows rapid recovery with efficient use of hospital resources.

In contrast, two cohort studies by Stottmeier *et al*[28] and Chen *et al*[27] highlighted that postoperative morbidity remains substantial after laparoscopic rectal surgery combined with early rehabilitation program, even though performed by experienced surgeons. Stottmeier *et al*[28] reported a median hospital stay of 5 d and a postoperative complication rate of 25% among 102 consecutive patients who had undergone elective fast-track laparoscopic rectal cancer surgery. Although about 40% of the patients had a diverting colostomy or ileostomy, reoperation was needed in 15% owing to anastomotic leakage, colonic ischemia, intra-abdominal abscess, or mechanical obstruction. Postoperative mortality (< 30 d) occurred in 3% of the patients; one with postoperative septicemia and pneumonia, one with postoperative multiorgan failure, and one with intraoperative splenic bleeding. Chen *et al*[27] calculated the success rate of their enhanced recovery program and reinvestigated factors that may have affected the results of the enhanced recovery program combined with laparoscopic rectal surgery. As designated by their program, patients were scheduled to be discharged on postoperative day 5. The criteria of discharge included absence of fever or tachycardia, successful passage of flatus or stool, tolerance of three meals per day, pain relief with oral nonopioid analgesics, and independent ambulation. They reported a success rate of 52.5%, and this failure was related to low rectal lesion sites (< 7 cm from the anal verge) and surgery-related complications, with a rate of 13.8%. The authors concluded that the enhanced recovery program for laparoscopic rectal surgery is feasible but is not advised for all cases requiring laparoscopic rectal surgery.

Previously, we had designed a prospective, randomized, controlled parallel group trial to compare the outcomes of an early rehabilitation program *vs* conventional care after laparoscopic low anterior resection in patients with mid-rectal or low rectal cancer (≤ 10 cm from the anal verge)[30]. The primary endpoint was recovery within 4 postoperative days and the criteria for recovery were as follows: tolerable diet for 24 h, safe ambulation, analgesic-

Table 3 Protocols used in previous studies for evaluating early rehabilitation programs after laparoscopic rectal surgery

Protocols	Lindsetmo *et al*[22], 2009	Chen *et al*[27], 2011	Stottmeier *et al*[28], 2012	Huibers *et al*[29], 2012	Lee *et al*[30], 2013
Preoperative stage					
General considerations	Patient education	Patient education and ERP explanation	Thorough information Establishing a contract	ND	Operative risk assessment Counseling, informed consent
Oral bowel preparation	Yes	Yes	No (enema for left-sided tumors)	No (2 enemas)	Yes
NPO	ND	8 h before surgery	Fluid until 2 h before surgery	2 h before surgery	8 h before surgery
Oral carbohydrate solution	No	No	No	Yes	No
Epidural analgesia	No	No	Yes	Yes	No
Prophylactic antibiotics	ND	Single dose	Single dose (ampicillin + metronidazole + gentamicin)	Single dose (cefalozine + metronidazole)	ND
DVT prophylaxis	ND	ND	LMWH 2 h before surgery Compression stockings	LMWH until discharge	ND
Perioperative stage					
Operation approach	Laparoscopic	Laparoscopic	Laparoscopic	Laparoscopic	Laparoscopic
Anesthesia	ND	Short-acting anesthetics	Propofol, remifentanyl and muscle relaxant	ND	ND
Fluid	ND	Perioperative fluid restriction	Avoid both hypovolemia and fluid overload	ND	ND
Urinary drainage	Urethral catheter	Urethral catheter	Suprapubic or urethral catheter	Urethral catheter	Urethral catheter
Nasogastric tube	Yes (orogastric tube, removed before extubation)	No	No	No	No
Intra-abdominal drain	Rarely	Yes	No	Yes (one)	Yes (one)
Postoperative stage					
Pain control	IV PCA (12-18 h) Ketorolac Oral analgesia	Oral NSAIDs immediately after surgery Opioid for 1 d if needed	Epidural analgesia Paracetamol, ibuprofen Opioid if needed	Epidural analgesia Paracetamol, diclofenac Opioid avoided	IV PCA till POD 2
Sipping water	Immediately after surgery	Immediately after surgery	Immediately after surgery	Immediately after surgery	Immediately after surgery
Oral food intake	POD 1	POD 1	Evening of the day of surgery	Liquid diet in the evening	Semi-fluid diet, POD 1
Removal of urinary catheter	POD 1	POD 1	Immediately after surgery	POD 2	POD 3
Removal of intra-abdominal drain	No drain	POD 4	No drain	POD 2	ND
Mobilization	As soon as possible	Immediately after surgery	Two hours after surgery	POD 1	POD 1
Regular laxatives	ND	Sennoside	MgSO4 1 g two dimes daily	MgO	MgO
Routine discharge	ND	POD 5	POD 3	ND	ND
Discharge criteria	Tolerance of fluids and solid diet, adequate oral analgesia, passage of flatus or stool, adequate home support	No fever, no tachycardia, successful passage of flatus/stool, tolerance for 3 meals/d, comfort in taking oral non-opioid analgesics, independent ambulation, adequate self-care ability	Adequate bladder and bowel function, ability to drink, eat, walk without problems, manageable pain	No remaining lines or catheters, toleration of solid food, passage of stool, controllable pain, self-care ability	ND (Recovery: tolerance of diet for 24 h, analgesic-free, safe ambulation, afebrile status without major complications)

ERP: Early rehabilitation program; DVT: Deep vein thrombosis; LMWH: Low-molecular-weight heparin; NSAID: Non-steroidal anti-inflammatory drug; PCA: Patient-controlled analgesia; POD: Postoperative day; ND: Not described.

free, and afebrile status without major complications. The sample size was based on a superiority design. All patients were between 20 and 80 years of age and had undergone temporary loop ileostomy with laparoscopic low anterior resection. Protocols for perioperative care programs and interventions were modified from previously described protocols for colonic surgery (Table 3). Ninety-eight patients were randomized on a 1:1 basis to an early rehabilitation or conventional care program. The recovery rates were no different in both groups; however, more com-

plications were observed in the rehabilitation program group (42.3% *vs* 24.0%, $P = 0.054$), which were related to postoperative ileus (28.8% *vs* 13.0%, $P = 0.057$), and acute voiding difficulty (19.6% *vs* 4.7%, $P = 0.032$). Our randomized trial did not show that an early rehabilitation program was beneficial after laparoscopic low anterior resection. These results support those of previous studies in that postoperative morbidity might be a major obstacle to the ERAS in rectal cancer surgery.

CURRENT EVIDENCE-BASED RECOMMENDATIONS FOR EARLY REHABILITATION AFTER RECTAL SURGERY

Consideration points for adopting early rehabilitation program in rectal surgery

For the successful application of early rehabilitation programs to patients undergoing laparoscopic rectal resection, we need to recognize that colon surgery is entirely different from rectal surgery, which requires a deep pelvic dissection and is frequently accompanied by higher complication rates, longer hospital stay, and associated with unique complications such as sexual dysfunction, urinary retention, and pelvic organ injury (*e.g.*, hypogastric nerves and ureters) not seen in intra-abdominal colonic resection. Compared with colonic segmental resection, rectal surgery has higher technical complexity, longer operative times, and use of retraction known to increase perioperative morbidity[8]. Therefore, previous studies involving early rehabilitation programs excluded patients undergoing rectal resection[1,3,4,8]. In some studies, the results of rectal resection were mixed in the overall analysis of the application of early rehabilitation program protocols[23,24,26,31].

The available guidelines for perioperative care in rectal surgery are currently limited[2,8]. Recently, guidelines for perioperative care in elective rectal surgery were published by the ERAS Society, which had also published colonic guidelines[8,16]. In these guidelines, the authors remarked that they specifically considered the application of ERAS principles to a special population of rectal resection patients, because of the differences between colonic and rectal surgery. Until now, ERAS Society recommendations seem to be the best evidence-based guidelines for each item of the perioperative treatment pathway. These recommendations were derived from extensive review of meta-analyses, randomized controlled trials, and large prospective cohorts. However, these guidelines are basically intended for open rectal surgery, and are not focused on laparoscopic surgery. ERAS Society recommendations assess the quality of evidence ("high", "moderate", "low", "very low"), and decide the strength of recommendations as follows: strong recommendations indicate that the panel is confident that the desirable effects of adherence to a recommendation outweigh the undesirable effects; and weak recommendations indicate that the desirable effects of adherence to a recommendation probably outweigh the undesirable effects, but the panel is less confident[8]. Many items in the recommendations are based on low or moderate level of evidence. Some items are recommended by a high level of evidence, such as prophylaxis against thromboembolism or preoperative bowel preparation; however, studies on these items are based on the results of patients undergoing open surgery or in a population undergoing both open and laparoscopic surgery. Specific validation for these items in patients undergoing laparoscopic rectal resection remains insufficient.

Currently, no early rehabilitation protocol perfectly fits all patients undergoing laparoscopic rectal surgery[2]. For each individual patient, these guidelines, which are suggestions on the basic concept for early rehabilitation, should be modified to optimize perioperative care, minimize postoperative morbidity, and improve overall patient outcomes.

Patient selection, counseling and risk assessment

The first step is selecting patients. Extensive discussion with candidate patients on the entire surgical procedure followed by early rehabilitation program may be the most important step. This step can give patients the best insight into the benefits and risks and motivate them to make an effort to enhance their recovery after surgery because the success of early rehabilitation is affected by the active participation of the enrolled patient[2]. Previous studies and guidelines recommended direct interview, leaflets, or multimedia as information-providing methods[8]. Generally, patients who are bedridden, severely malnourished, and with an American Society of Anesthesia (ASA) score ≥ 3, who are planning to receive emergency rectal surgery are excluded, and any healthy patients with ASA 1-2 are included[8,32]. It is also important to improve the patient's medical condition by correcting anemia, malnutrition, or hyperglycemia, and promoting cessation of smoking and alcohol consumption at least 4 wk before surgery[33].

Bowel preparation

Mechanical bowel preparation (MBP) is considered a necessary step before colorectal surgery, and it is believed to decrease the risk of infectious complications and anastomotic leakage. However, several studies, including large meta-analyses, showed no difference between the MBP and no MBP groups on infection rates or anastomotic leakage after colorectal surgery[8,34-36]. Some studies suggested that MBP increased dehydration and electrolyte imbalance[37]. On the contrary, a recent multicenter randomized trial showed that overall and infectious complications were higher in the no MBP group compared with the MBP group in patients undergoing low anterior resection. In this study, a non-significant trend to a two-fold higher risk of anastomotic leak (19% in no MBP *vs* 11% in MBP) was also observed[38]. Current guidelines support omitting MBP in colonic surgery but indicate insufficient evidence supporting this omission in rectal

surgery[8,39,40]. There has been no study on MBP efficacy in the context of early rehabilitation programs. The Society of American Gastrointestinal and Endoscopic Surgeons Guidelines comments that MBP may be helpful in laparoscopic colorectal surgery, because it can make laparoscopic colorectal manipulation easier[40]. Further studies comparing MBP with no MBP in patients undergoing laparoscopic rectal surgery are necessary.

Postoperative pain

Postoperative analgesia is critical to enhance patient recovery because it directly affects early ambulation and patients comfort. Postoperative analgesia requires a multimodal approach consisting of the collaboration of the patient, surgeon, nurse, anesthesiologist and pain specialist[2]. Patient-controlled opioid analgesia (PCA) usually shows satisfactory result after rectal surgery[41]. However, PCA has some side effects influencing early recovery of patients, such as nausea, vomiting, and prolongation of postoperative ileus as well as sedation and respiratory suppression[2].

Two recent guidelines recommended continuous epidural analgesia (CEA) for open rectal surgery during 48-72 h, with intravenous administration of lidocaine in view of the superior efficacy of pain relief compared with systemic opioids[2,8,42]. CEA has the benefit of delivering a combination of local and opioid analgesia directly to the dorsal horn of the spinal cord, thus providing pain relief without systemic opioid effects[43]. However, this method involves an invasive procedure for catheter insertion and has some side effects, including pruritus, urinary retention, and arterial hypotension[44]. Some authors have advocated CEA use in the context of early rehabilitation in patients without contraindications[45,46]. They have suggested that the superiority of CEA seems to be greatest in the first 2-3 d postoperatively, and thus, routine removal of CEA after 2 or 3 d postoperatively may be a useful strategy. Some studies have shown that, in laparoscopic approaches that use only several small incisions instead of a single, large vertical incision from the umbilicus down, continuous intravenous infusion of lidocaine or PCA, as alternatives for CEA, also provide good pain relief in the first 24 h with a similar time to return of bowel function or length of hospital stay[8,47].

Pelvic drainage

The use of pelvic drainage after low anterior resection has been a controversial issue in rectal surgery. Some surgeons still prefer insertion of a drain into the pelvic cavity to prevent bloody ascites and its adverse effect on anastomosis. Several randomized trials and meta-analyses have shown that the routine use of a pelvic drain does not affect the anastomotic leakage or overall complications[48-50]. However, the use of a drain should be considered in cases of clinical indications, such as high-risk individuals or suspicion of tenuous anastomosis[8].

Prevention of ileus

Prevention of postoperative ileus is a crucial element not only for success of early rehabilitation, but also postoperative morbidity, readmission, and overall outcomes. To promote bowel motility after abdominal surgery, several methods have been evaluated, including gum chewing, oral magnesium oxide, and bisacodyl suppositories[51-54]. These methods have been reported to reduce time to bowel movement by 1-2 d, but there was no effect in the length of hospital stay or overall outcomes. However, the association of these medications with anastomotic dehiscence has not been addressed in a randomized trial of sufficient size. Furthermore, anastomotic leakage and temporary stoma should be considered in the use of stimulant laxatives after rectal surgery. Ileostomy has been reported as an independent risk factor for postoperative ileus, which developed in 22.8% of patients[55]. Our previous randomized controlled trial to evaluate the efficacy of an early rehabilitation program after laparoscopic rectal surgery also indicated a similar result, showing that a rehabilitation program introducing an early oral diet could increase postoperative ileus. Thus, further studies are necessary[30].

CONCLUSION

Early rehabilitation combined with laparoscopic rectal surgery is a feasible alternative in some selected patients, but indications have not been established. Current evidence fails to support the safety of early rehabilitation combined with laparoscopic rectal surgery compared to that reported for laparoscopic colonic surgery. Long-term outcomes, which might be affected by postoperative complications, in patients with malignant disease are unknown after laparoscopic rectal surgery followed by an early rehabilitation program. More data from well-designed clinical trials should be accumulated for widening the adoption of early rehabilitation programs to patients undergoing laparoscopic rectal surgery.

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REVIEW

Pathophysiology of cystic fibrosis and drugs used in associated digestive tract diseases

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Abstract

Cystic fibrosis (CF) causes chronic infections in the respiratory tract and alters the digestive tract. This paper reviews the most important aspects of drug treatment and changes in the digestive tract of patients with CF. This is a review of the literature, emphasizing the discoveries made within the last 15 years by analyzing scientific papers published in journals indexed in the Scientific Electronic Library Online, Sciences Information, United States National Library of Medicine and Medical Literature Analysis and Retrieval System Online databases, both in English and Portuguese, using the key words: cystic fibrosis, medication, therapeutic, absorption, digestion. Randomized, observational, experimental, and epidemiological clinical studies were selected, among others, with statistical significance of 5%. This review evaluates the changes found in the digestive tract of CF patients including pancreatic insufficiency, constipation and liver diseases. Changes in nutritional status are also described. Clinical treatment, nutritional supplementation and drug management were classified in this review as essential to the quality of life of CF patients, and became available through public policies for monitoring and treating CF. The information gathered on CF and a multi professional approach to the disease is essential in the treatment of these patients.

Key words: Cystic fibrosis; Medication; Therapeutic; Absorption; Digestion

Core tip: Cystic fibrosis (CF) has been studied in Brazil and in many other countries. Digestive manifestations may significantly compromise the nutritional status of CF patients, leading to numerous symptoms. Supplementation with enzymes, vitamins and nutrients is usually necessary. When infections are present, antibiotics are necessary, and these infections are often multisystemic, involving the digestive tract. The pharmaceutical assistance included in public policies, especially those which are financed, and the constant incentive to study the digestive manifestations in CF patients are essential, as without them, there would be infinite clinical changes which would compromise patient survival.

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INTRODUCTION

Cystic fibrosis (CF) is a chronic progressive disease, it exists in every ethnic group and it is equally common in both sexes. The CF gene has been isolated, cloned and sequenced, enabling the study of biochemical mechanisms responsible for the physiopathogenesis of the

disease. It also enables easier treatment of the patient's complications, such as the thick and viscous fluids which obstruct the lungs, the pancreas and the biliary duct[1,2].

The prevalence of CF varies according to ethnicity, from 1/1800 to 1/5000 in Caucasians born alive in Europe, in the United States and in Canada, 1/14000 in Afro-Americans, and 1/40000 in Finland. It is considered a rare disease among Asians and Africans. In Brazil, local studies show variable statistical data which suggest an approximate incidence of 1/7000. The average lifetime of CF patients has increased in the last few years, which is the result of early diagnosis and specialized treatment in the early stages of the disease[1,3,4].

The treatment of CF aims to clear the lungs using aerosols and respiratory physiotherapy, and to maintain nutritional status with nutrient supplementation and pancreatic enzymes. Recent medical advances have improved survival, but with increased costs, especially when the disease has progressed and when hospitalization is required. When infections are present, antibiotics are necessary, usually due to clinical complications which are often multisystemic, and involve the digestive tract[5,6]. Due to many involved systems and the variety and chronicity of the disease, a multitask approach is essential to help the patients and their families to comprehend the disease and undergo medical treatment[7].

The current therapy for CF includes the maintenance of nutritional status, clearance of the pulmonary tract, utilization of antibiotics and other medication, treatment and monitoring of gastric, pancreatic and hepatobiliary changes, in addition to dietary supplementation with hypercaloric and hyperproteic foods, and the utilization of enzymes, minerals and vitamins[1,8,9].

When chronic CF is diagnosed, with many clinical manifestations, the continuous use of medication (antibiotics, bronchodilators, mucolytics) and related procedures (respiratory physiotherapy, oxygen therapy, lung transplantation, digestive enzyme replacement and nutritional support) are required[8,10]. Due to the chronicity and the need for precautions in CF, the development of a Reference Center and the establishment of an organization that involves family members is crucial, together with an increase in cooperation between groups of CF patients and other organizations[4,11,12].

CF requires the continuous use of medication which increases the average cost of treatment, and is too expensive for families. For that reason, CF patients and their families have the right to receive government help under the Unique Health System. The clinical record of the Health Ministry guarantees access to alpha dornase for pulmonary complications and pancreatic enzymes in patients with pancreatic insufficiency[3]. There are many deeds in every unit of the federation, including the Distrito Federal, to promote early diagnosis and even provide special formulas such as the alimentary supplements provided by Ordinance number 94/1809, published at the Distrito Federal in 2009[13].

In Brazil, the dedication to diagnosing CF during infancy is significant, with the use of programs for newborn screening or sweat testing. It is known that early treatment, including drug treatment, contributes to the prognosis and survival of CF patients[14-17].

The objective of this study was to review the most important aspects of drug treatment and changes in the digestive tract of patients with CF. We also aimed to assess the pharmaceutical monitoring offered to CF patients undergoing treatment by public agents from the public health care system.

REVIEW OF LITERATURE

This review focused on CF literature over the last 15 years, and included scientific papers indexed in the databases of Scientific Electronic Library Online, Sciences Information, United States National Library of Medicine and Medical Literature Analysis and Retrieval System Online, using the key words: cystic fibrosis, medication, therapeutic, absorption, digestion. Studies in English and Portuguese were selected.

The survey focused on the major advances in the understanding of CF during this period, both in understanding the disease and its treatment.

Articles that included at least one of the mentioned key words were selected. Controlled clinical studies were included, as well as observational epidemiological studies and meta-analyses, among others. Papers which did not include information on the diagnosis of CF or adherence to treatment were excluded as were experimental animal studies and gene therapy studies and those published in languages other than English and Portuguese.

RESULTS AND DISCUSSION

Physiopathology of the disease: overall symptoms

The manifestation of CF is very changeable and may appear in the neonatal period or later in life. Some patients are completely asymptomatic for several years. The most common clinical signs of CF include a chronic cough, chronic diarrhea and malnutrition; however, the disease can appear in other ways, and can affect multiple systems and organs[18].

Mutation of the *CF* gene causes absence or dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR) protein, which works as a chloride canal in the apical membranes of epithelial cells. The CFTR also affects the production of mucus, secretory granules and intracellular organelles. This defect affects cells in many organs, not all organs have similar clinical responses, and different organs may be affected. Involvement of the respiratory tract is associated with a higher death rate and leads to death in 90% of patients[18-20].

The most common and important symptom which affects the digestive tract is exocrine pancreatic insufficiency, characterized by chronic diarrhea with undigested food present. A decrease in the secretion of sodium bicarbonate reduces the efficacy of pancreatic enzymes

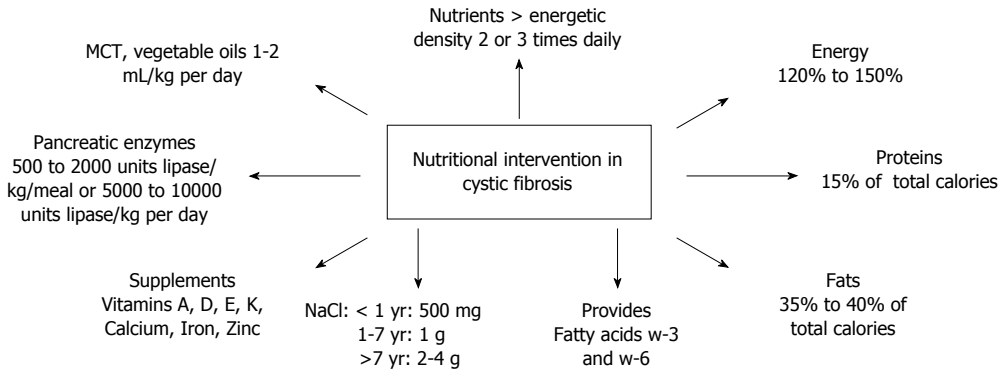

Figure 1 Macronutrients and micronutrients essential for the recovery and maintenance of nutritional status in cystic fibrosis patients[7]. MCT: Medium chain triglycerides.

and the precipitation of bile salts, which results in a more acidic pH in the duodenum, contributing to malabsorption[18].

The obstruction of pancreatic canaliculi by mucous plugs prevents the release of enzymes into the duodenum, which causes poor digestion of fat, proteins and carbohydrates. Malabsorption is caused by pre-epithelial dysfunction, which occurs after the rejection of non-hydrolysable nutrients in the lumen. Therefore, malnutrition occurs due to inadequate food digestion and increased energy needs (dietary recommendations) that are rarely achieved by CF patients due to anorexia and recurrent respiratory disease among other diseases[18,21-23].

The endocrine pancreas also undergoes changes and the prevalence of CF related to glucose intolerance has increased proportionally with the rate of survival. The main cause of diabetes is damage caused to the pancreas, leading to a decrease in insulin secretion. Diabetes in CF patients results from microvascular and macrovascular complications associated with accelerated lung deterioration, consequently increasing the death rate. Since nutrition is critical in CF patients, blood glucose should be monitored and the insulin dose should be adapted, with a focus on adequate intake of nutrients[24].

Symptomatic vitamin A and vitamin E deficiency has been reported in patients with CF presenting with deficit nutrient consumption and absorption[25,26].

Many newly diagnosed infants have low levels of one or more fat-soluble vitamins[27,28] and due to the prevalence of fat-soluble vitamin deficiency, all infants with CF should receive standard, age-appropriate non-fat-soluble vitamins and vitamins A, D, E, and K as recommended in the CF Foundation Consensus Report on Nutrition for Pediatrics[29].

Most patients who are vitamin deficient can be treated adequately with the doses of fat-soluble vitamins recommended in the CF Foundation Consensus Report on Nutrition for Pediatric Patients[30].

Figure 1 shows relevant information on the nutritional care of CF patients[7].

Among other events related to CF, meconium ileus, obstruction of the terminal ileum by thick meconium, is the first signal of pancreatic insufficiency, which affects 15% of babies. Therefore, treating patients with meconium ileus is very important until proved otherwise[31].

Early diagnosis and the treatment of complications of the respiratory and gastrointestinal tract in CF can lead to an improvement in the survival rate of CF patients. Those who live beyond the fourth decade have a higher risk of developing additional diseases associated with chronic manifestations; hence, patients with a higher risk of chronic diseases should be monitored closely to improve the chances of early diagnosis[32].

Figure 2 summarizes the majority of abnormalities observed in the digestive tract of patients diagnosed with CF from intrauterine life to adulthood.

Gastrointestinal disease

In CF patients gastrointestinal symptoms, such as nausea, vomiting, malnutrition and indigestion are frequent. In addition, gastroesophageal reflux disease, esophageal adenocarcinoma, distal intestinal syndrome and cholelithiasis are often seen in CF patients[33-35].

There is increasing evidence to suggest that chronic inflammation is present in the gastrointestinal tract of CF patients. Some CF patients continue to have many severe gastrointestinal symptoms despite conventional CF treatment[36].

A recent publication indicated the presence of eosinophilic esophagitis (EoE) in CF patients aged from 4, 12 and 15 years. Patients with CF may have clinically persistent emesis, food aversion and failure to thrive. It is possible that EoE has been underappreciated in CF due to symptom overlap with other common gastrointestinal disorders, including gastroesophageal reflux disease, infections, medication side effects or others conditions[37].

Because the symptoms in EoE are non-specific and are also common in CF, when a patient with CF presents with food avoidance, regurgitation, heartburn or dysphagia, EoE should be considered, particularly if symptoms do not respond to empiric treatment and if endoscopic evaluation is contemplated[38-40].

Secretory cells of CF patients show modification in their absorptive-digestive function in the gastrointestinal tract and the entire digestive process is altered, which results in malabsorption of nutrients, malnutrition and

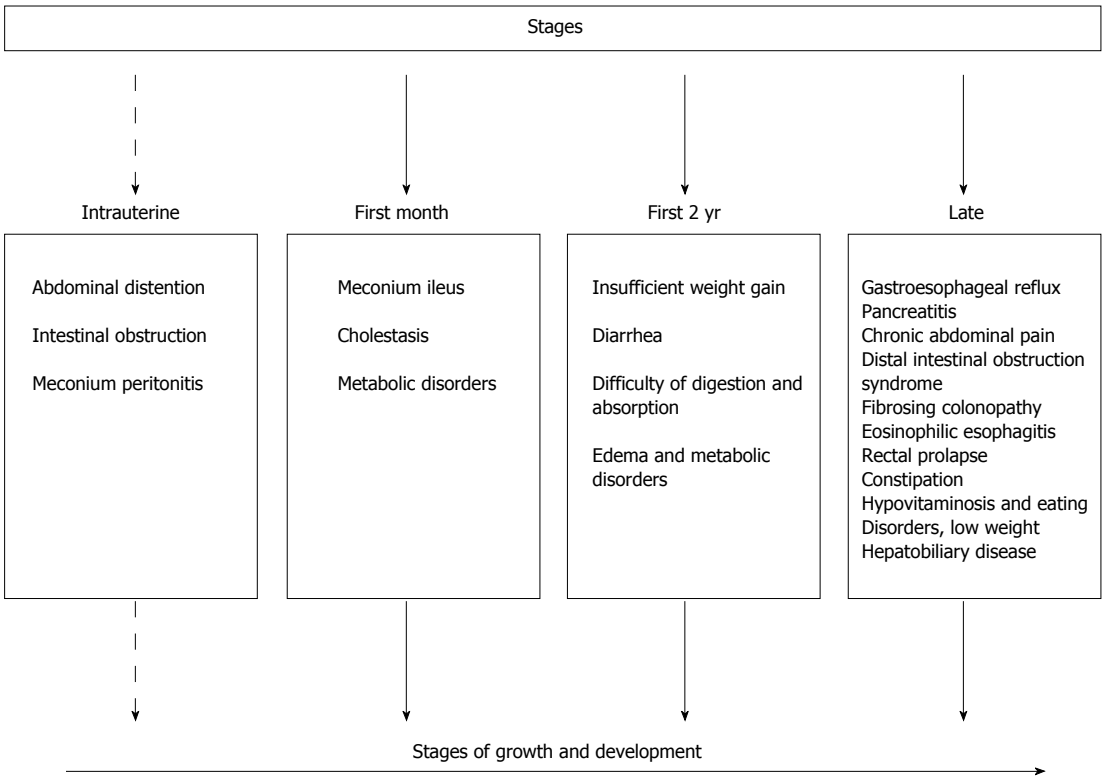

Figure 2 Summary of major abnormalities observed in the digestive tract of patients diagnosed with cystic fibrosis from intrauterine life to adulthood.

several gastrointestinal tract-related symptoms[34,41].

Abdominal pain is a common complaint in CF patients, and distal bowel obstruction syndrome and fibrosing colonopathy are characteristics of gastrointestinal complications in CF patients. The main causes of epigastric pain in patients with CF are gastroesophageal reflux disease, biliary tract disease, pancreatitis and gastritis[42,43].

Among the frequently observed gastrointestinal manifestations, gastroparesis has been diagnosed by a variety of methods and has been described by CF patients. Gastroparesis is a frequent complication of lung or heart-lung transplantation. It is predominantly found in children and individuals with severe deterioration of the pulmonary tract[43,44].

After meconium ileus, the main area affected by distal bowel obstruction syndrome (DIOS) is the right colon. DIOS is more common in patients with pancreatic insufficiency. Several factors can trigger the syndrome, such as dehydration, the use of medicines which interfere with intestinal motility and pancreatic enzyme replacement. The most common signs and symptoms of DIOS are decreased defecation and colic pain in the right lower quadrant. During clinical examination, a reduction in intestinal peristalsis can be observed, with the possibility of cessation at some point. In some cases, a mass in the lower right quadrant can be palpated, which is related to distention of the cecum and right colon[45].

Intestinal obstruction syndrome is similar to meconium ileus; however, one of the differences between these conditions is patient age. Intestinal obstruction syndrome is characterized by the impaction of fecal residues in the terminal ileum and one of the precipitant factors for obstruction is dehydration. This obstruction can be total or partial, and may cause symptoms such as abdominal distention, constipation, anorexia, vomiting, and early satiety, which result in weight loss[45].

Fibrosing colonopathy is another characteristic of CF, and includes a change in the colon submucosa, inflammation, and progressive fibrosis associated with managing the high doses of pancreatic enzymes. The clinical symptoms are pain and abdominal distention after ingesting food, anorexia, difficulty in gaining weight and digestive bleeding[34,46].

Pancreatic disease

The pancreas is one of the main organs affected by dysfunction of the CFTR. The exocrine pancreas is responsible for producing enzymes for food digestion in the intestinal lumen and exocrine pancreatic insufficiency is a well-known complication of CF and leads to fat loss in feces. Loss of function of the pancreas is associated with every genotype of CFTR mutation, leading to pancreatic insufficiency[47-49].

Pancreatic exocrine insufficiency (PEI) is considered the main cause of intestinal malabsorption in CF, affecting 85% to 90% of patients[50], and if inadequately treated high stool energy losses will occur, which is an important determinant of energy imbalance and malnutrition[51].

Intestinal malabsorption is usually of early onset: signs and symptoms of maldigestion are often present at birth, and in the majority of patients, during the first years of life. At the time of diagnosis, at least 50% of in-

Table 1 Fat-soluble vitamins used for supplementation in cystic fibrosis patients[1]

Vitamins	Dosage	Dosage
A	400-10000 UI (approximately 2240 μg)	Daily
D	400-1800 UI (approximately 18 μg)	Daily
E	50 mg (1 yr) 100 mg (1- 10 yr) 180 mg (adolescents and adults)	Daily
K	0.3-0.5 mg	Daily

fants identified by neonatal screening have PEI, and most of those carrying severe CFTR mutations on both alleles develop PEI during the first years of life[52-55].

PEI is clinically characterized by weight loss or difficulty in gaining weight, diarrhea with a greasy appearance and malabsorption of fat-soluble vitamins A, D, E and K. Thus, the supplementation of these vitamins is routinely recommended, followed by blood examinations to manage the dose and the correct nutrients according to the patient's needs[27,56-58].

Vitamin D is of great interest in CF due to its role in bone mineralization and its deficiency has been hypothesized to play a role in the development of depression. Hypovitaminosis is almost universal in patients with CF. Insufficient levels are widely reported and is associated with increasing age and obesity. Vitamin D screening and supplementation should be considered in all children with chronic illness, particularly those who are overweight[59-62].

Table 1 shows treatments with fat-soluble vitamin supplementation in CF patients[1].

Hepatobiliary disease

The primary hepatic changes in CF involve a genetic defect in the CFTR protein, leading to the production of a thick biliary secretion, followed by biliary fibrosis[34]. Cirrhosis, ascites, portal hypertension, esophageal varices and bleeding are complications of hepatobiliary disease associated with CF, and frequently affect teenagers and adults[33].

This dysfunction is predicted to result in defective (sluggish) bile flow, and is associated with a cholangiocyte-induced inflammatory response with activation and proliferation of hepatic stellate cells, which results in cholangitis and fibrosis in focal portal tracts[63-66].

Approximately 5%-10% of CF patients develop multilobular cirrhosis during their first decade of life. Subsequently, most tend to develop signs of hypertension with complications, especially variceal bleeding. Annual examinations are recommended to detect hepatic disease, and when presymptomatic signs are present therapy with ursodeoxycholic acid is recommended, which can prevent disease progression[67,68].

Cystic fibrosis-related liver disease (CFLD) is defined if at least 2 of the following conditions are present on at least 2 consecutive examinations spanning a 1-year period: (1) Ultrasound confirmed hepatomegaly; (2) Elevated serum levels of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, gamma-glutamyltransferase; and (3) Ultrasound abnormalities other than hepatomegaly (*i.e.*, increased, heterogeneous echogenicity, nodularity, irregular margins, splenomegaly). An ultrasonographic pattern of simple liver steatosis does not represent a diagnostic criterion. In the case of distinct ultrasonographic signs of liver cirrhosis (*i.e.*, coarse nodularity, presence of portal hypertension and rarefaction of peripheral portal veins) and clinical signs (*e.g.*, esophageal varices, splenomegaly) of liver cirrhosis, CFLD patients are classified as cirrhotics[63,69].

Liver disease can only be taken into consideration if the physical examination is abnormal and abnormal hepatic function persists, and the latter has to be proved using ultrasound. If there are any doubts, a liver biopsy is suggested. All patients with liver disease require to be monitored annually to evaluate the progress of hypertension, portal cirrhosis or liver failure. Prophylactic measures for liver disease are nutrition monitoring, bleeding prevention and variceal decompression. In liver transplantation, deterioration of the organ has to be taken into consideration, especially in children with hepatic dysfunction or advanced hypertension[68].

Treatment

Treatment with pancreatic enzymes in patients with pancreatic insufficiency is associated with an increase in the coefficient of fat absorption, a decrease in bowel movement frequency, an improvement in the consistency of feces and weight gain. One of the aims of pancreatic enzyme replacement therapy is to abolish unpleasant gastrointestinal symptoms[45].

The response to treatment is individually evaluated, and doses are adjusted according to nutritional status. The use of antacids is recommended in patients taking enzymes to increase bioavailability, although, there is insufficient evidence to indicate whether there is an improvement in quality of life or survival[1,70].

In young children whose fat intake is known to vary with age, particular attention needs to be paid to fat malabsorption during pancreatic enzyme supplementation. More importantly, young children often have difficulty swallowing the available enzyme formulations, which may lead to suboptimal compliance and treatment effects[71].

The initial dose of pancreatic enzymes can be calculated based on the weight of the patient taking into consideration the dietary fat intake. 500 to 1000 U of lipase/kg is administered per main meal, the dosage can be increased according to clinical signs, and the maximum daily dose should not exceed 2500 U/kg per meal or 10000 U/kg per day of lipase[3].

Figure 1 summarizes pancreatic enzyme dosage[7].

The guidelines recommend that if dose increases are required, they should be increased with careful monitoring of body weight and stool fat content. When controlled clinical trials are designed to assess the safety and efficacy of pancreatic enzyme replacement therapy, the dose in terms of lipase units is usually limited to a level

within the recommended range. However, in everyday clinical practice it is possible that maldigestion is not adequately controlled by the recommended doses in a proportion of CF patients: these patients may, therefore, require higher lipase doses[72-74].

The United Kingdom Cystic Fibrosis database indicates that lipase dose often exceeds 10000 U/kg per day for extended periods in clinical practice, both with standard-dose and high-dose pancreatic enzyme preparations. These high-dose regimens appear to have good safety and tolerability profiles, and fibrosing colonopathy has not been reported in recent years. However, it is essential that the safety and efficacy of higher doses of pancreatic enzyme replacement therapy are fully explored, particularly in the long-term, clinical practice setting[74,75].

There are several options for the treatment of EoE, including pharmaceutical agents and dietary elimination. Consensus recommendations advocate first-line treatment with oral corticosteroids (*e.g.*, fluticasone, budesonide) or dietary therapy depending on patient preference and illness severity[38].

Dietary therapy can be very effective in children if culprit food allergens are identified, and recent data show this to be effective including the elimination of offending agents (targeted elimination diet), or an allergen-free diet consisting only of an elemental formula (elemental diet)[76-78].

The correction of steatorrhea is essential in CF. In the past, diets low in fat were recommended to try to reduce steatorrhea. Currently, restrictive diets have been replaced by hypercaloric diets rich in fat, which is a source of energy, are more economical and their intake should be encouraged[79]. The dose and timing should be followed very strictly, and patients should adhere to treatment. For infants, apple juice or small quantities of milk are consumed, and meals should be carried out in block in order to benefit from the bioavailability of the entire quantity of administered enzyme[49].

Medium chain triglyceride fats should be included in the standard dietary regimen used in the management of any child with CF and failure to thrive. Their use is fully justified due to clinical improvement and alleviation of steatorrhoea[80].

In clinical practice, probiotics have been frequently prescribed for patients suffering from diarrhea to protect the body against pathogens[81].

A probiotic is a "live microbial food ingredient that, when ingested in sufficient quantities, exerts health benefits on the consumer". Probiotics exert their benefits through several mechanisms; they prevent colonization, cellular adhesion and invasion by pathogenic organisms. The strongest evidence for their clinical effectiveness has been in their use for the prevention of symptoms of lactose intolerance, treatment of diarrhea, and attenuation of antibiotic-associated gastrointestinal side effects[81].

Probiotics reduce the rate of pulmonary exacerbations in patients and may have preventive potential for pulmonary deterioration in CF patients[82-84].

To ensure a continuous effect, probiotics and prebiotics need to be ingested daily. Favorable changes in the composition of intestinal microbiota were observed at doses of 100 g of food product containing 109 colony forming units (cfu) of probiotic microorganisms and doses of 5 to 20 g inulin and/or oligofructose, usually during the administration period of 15 d. Thus, to be of physiological importance to the consumer, probiotics must reach populations greater than 106 to 107 cfu/g or mL bioproduct[85].

The goal of nutritional therapy is to maintain the ideal weight, reduce malabsorption and digestion and control the intake of vitamins and minerals[1]. CF patients require diets with a high energetic rate (120% to 150% of the regular daily need for weight, height and age), hypercaloric, high-fat and high protein, divided into 5-6 meals a day and supplemented with vegetable oils such as medium chain triglycerides. In cases where dietary treatment does not result in weight gain, the diet can be offered in small volumes, several times a day or administered in the evening of through a nasogastric tube or gastrostomy. Enteral tube feeding has been evaluated in pediatric and mixed child and adult populations with CF, demonstrating positive outcomes post-insertion. The diet may be administered through an infusion pump or gravitational and it is recommended that the night diet reaches 40%-50% of the daily energy requirements so that there will be recovery or maintenance of the nutritional state[7,86,87].

CF may include intestinal inflammation and CF patients have altered fatty acid metabolism characterized by an imbalance in the arachidonic/docosahexaenoic acid ratio in favor of the former, which can contribute to an increase in inflammation. Recent studies indicate that changes in fatty acid metabolism are responsible for abnormalities, and dietary supplementation with fish oils high in the omega-3 fatty acids, eicosapentaenoic acid and docosahexaenoic acid may have an anti-inflammatory effect[88-91].

Various anti-inflammatory therapies, including dietary omega-3 polyunsaturated fatty acids supplementation, have been investigated in CF patients. The composition of dietary omega-3 and omega-6 influenced the inflammatory markers in CF and dietetic integration seems to improve clinical condition and the inflammatory pulmonary and intestinal state in patients suffering from CF[92,93].

With a partial bowel obstruction, intestinal disimpaction is stimulated by hypertonic solutions, such as *N*-acetylcysteine, polyethylene glycol or hypertonic contrast, orally or by using probes. In cases of total obstruction the disimpaction is performed through enemas, while keeping the patient hydrated. After the disimpaction, pancreatic enzyme treatment should be included in the preventive treatment in obstructive conditions, administering lactulose, mineral oil, polyethylene glycol or N-acetylcysteine to the patient. Prokinetic drugs may also be helpful[34].

It is recommended that, in the case of fibrosing colonopathy, there is a reduction in the enzyme dose associated with nutritional support with either semi-elemental or elemental formulas according to the evaluation by the nutritionist for nutritional enteral therapy, and if necessary, associated with parenteral nutrition in the most severe cases. In the case of digestive bleeding, a surgical procedure is prescribed[34,46].

The treatment of liver diseases focuses mainly on preventing disease progression which follows the sequence of cholestasis, fibrosis and cirrhosis. The maintenance of nutritional status is a part of this treatment, and aims to achieve and maintain the ideal weight of the patient, reduce malabsorption and maldigestion and control the intake of vitamins and minerals. However, nutritional treatment consists of enzyme replacement therapy, hypercaloric, high fat and micronutrient supplementation diets[1].

Supplementation with taurine has also been suggested to improve the solubilization of lipid micelles by bile acids. Taurine is a conditionally essential amino acid that possibly improves the micellar phase of fat digestion. Patients with CF and severe steatorrhea, despite appropriate enzyme therapy, showed a significant improvement in the absorption of triglycerides, total fatty acids, and linoleic acid while receiving taurine supplements. Taurine supplementation could be a useful adjunct in the management of patients with CF with ongoing fat malabsorption and essential fatty acid deficiency[94,95].

If CF patients also have taurine deficiency, this will result in malabsorption of bile acid and will require treatment with ursodeoxycholic acid (UDCA). The use of UDCA can increase the need for taurine administration for conjugation of bile acid[33].

UDCA is the drug currently used in CF patients and aims to slow the progression of liver disease. UDCA is a hydrophilic drug and is not significantly concentrated in bile. It has a hepatoprotective effect with rare collateral effects reported[33] and is frequently used in CF. UDCA inhibits the hepatic synthesis of cholesterol and promotes the synthesis of bile acids, thereby restoring the necessary balance between cholesterol and bile salts. The suggested dose is 14-18 mg/kg per day, 2 to 3 times a day up to 30 mg/kg per day[3,96].

Although it is one of the therapeutic options currently used for early changes in the liver, the use of UDCA as a preventive method requires further investigation as there are insufficient data on its long-term use, although adverse effects are rarely reported[97].

Liver transplantation may be necessary in patients with progressive liver failure and/or evidence of major portal hypertension in the absence of significant pulmonary involvement[98,99].

Careful monitoring and treatment should be offered to patients with CF associated liver disease (CFALD) and portal hypertension as they may require supplemental feeding by gastrostomy. However, this could lead to the development of stomal varices, which is an unwanted complication. A recent study evaluated the risk of gastrostomy in a series of seven children with CFALD and portal hypertension. The research concluded that gastrostomy placement for poor nutrition in children with CFALD and portal hypertension is safe and contributes to improved nutritional and pulmonary outcome[100].

CF is a multisystem disease and therefore requires different input from different professional reference centers for the treatment and monitoring of CF, supported by public health policies.

CONCLUSION

CF has been extensively studied in Brazil and many other countries. Digestive manifestations significantly compromise the nutritional status of the patient and lead to numerous symptoms, organ deterioration, the need for transplantation and resections which can worsen the multisystem disease.

Reference Centers with up-to-date medical teams to monitor and treat CF patients and initiatives such as the Brazilian Cystic Fibrosis Research Group can contribute to the dissemination and standardization of information, in addition to improving the quality of treatment.

The scientific literature contains an important variety of drugs, including many that are available without charges through programs from the Unique Health System, Brazil.

The pharmaceutical assistance and the constant incentive to study digestive manifestations in CF patients are essential, as without them, there would be infinite clinical changes that would compromise patient survival.

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REVIEW

Endoscopic tools for the diagnosis and evaluation of celiac disease

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Abstract

Celiac disease (CD) is an autoimmune disease of the small bowel induced by ingestion of wheat, rye and barley. Current guidelines indicate histological analysis on at least four duodenal biopsies as the only way to diagnose CD. These indications are based on the conception of the inability of standard endoscopy to make diagnosis of CD and/or to drive biopsy sampling. Over the last years, technology development of endoscopic devices has greatly ameliorated the accuracy of macroscopic evaluation of duodenal villous pattern, increasing the diagnostic power of endoscopy of CD. The aim of this paper is to review the new endoscopic tools and procedures proved to be useful in the diagnosis of CD, such as chromoendoscopy, Fujinon Intelligent Chromo Endoscopy, Narrow Band Imaging, Optical Coherence Tomography, Water-Immersion Technique, confocal laser endomicroscopy, high-resolution magnification endoscopy, capsule endoscopy and I-Scan technology.

Key words: Celiac disease; Malabsorption syndrome; Duodenum; Diagnostic techniques and procedures; Endoscopy; Chromoendoscopy; Fujinon intelligent chromo endoscopy; Narrow band imaging; Optical coherence tomography; Water-immersion technique; Confocal laser endomicroscopy; High-resolution magnification endoscopy; Capsule endoscopy; I-scan technology

Core tip: Celiac disease (CD) is an autoimmune disorder induced, in genetically predisposed people, by the ingestion of proteins rich in proline and glutamine. The aim of this review is to focus on the new endoscopic tools and techniques developed over the last years which can be useful in the diagnosis and the follow-up of CD.

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INTRODUCTION

Celiac disease (CD) is an autoimmune disorder induced, in genetically predisposed people, by the ingestion of proteins rich in proline and glutamine. It occurs in adults and children with an average prevalence of about 1% of the population. CD is characterized by an inflammatory reaction, primarily in the upper small intestine, with features of infiltration of the lamina propria and the epithelium with chronic inflammatory cells and progressive villous atrophy[1,2]. At the state of the art the role of serology is becoming more and more important, so that, according to the European Society for Paediatric Gastroenterology, Hepatology, and nutrition guidelines, diagnosis of celiac disease can be performed without histology in some selected situations-such as the presence, in children, of human leukocyte antigen-DQ2, high titers of

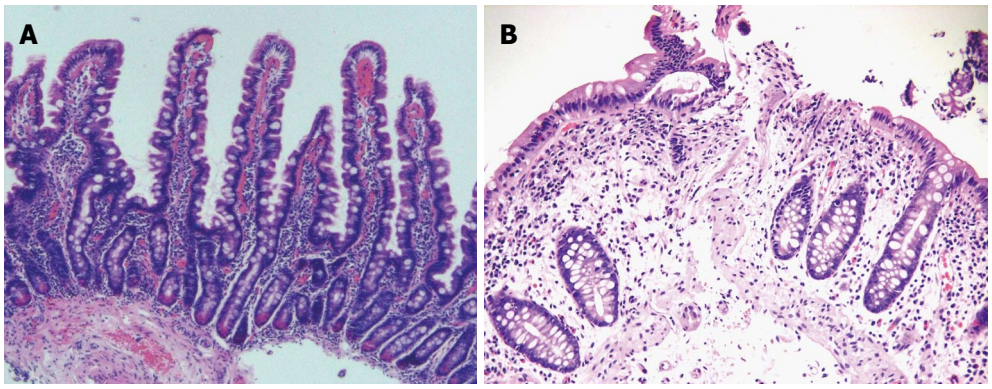

Figure 1 Histological appearance respectively. A: Normal duodenal pattern; B: Celiac disease.

anti-tissue transglutaminase antibodies and the positivity of anti-endomysial antibodies[3]. However, current guidelines indicate histological analysis as the gold standard for the diagnosis of CD: specific pathological features are infiltration of the lamina propria, crypt hyperplasia and villous atrophy, classified according to the Marsh classification and its modifications[4-8] (Figure 1). To perform a correct diagnosis, biopsy specimens have to be well oriented, and of good quality. From 4 to 6 duodenal biopsies, including a bulb biopsy, are required to make diagnosis of CD, even because villous atrophy can be unequally distributed -that is the so-called "patchy atrophy"[7,9-13].

Anyway, the diagnosis of CD can also be missed if the disease is not suspected and biopsy sampling not performed. So, in such situations, the role of the endoscopist becomes crucial, because of the strong importance of the macroscopic appearance of the duodenum[14-16].

STANDARD ENDOSCOPIC FINDINGS

A number of macroscopic endoscopic markers of CD has been identified over the years, and they include the following: "scalloping" -that is a dented aspect- of the duodenal folds; an absence or a reduction in number of duodenal folds; evidence of submucosal vascular pattern; the so-called "mosaicism", which is a micronodular look of the mucosa; finally, grooves and fissurations of the mucosa[9-10,14,15]. Results about the value of these markers, however, are conflicting: among different studies, the overall specificity and sensitivity sways from 83% to 100%, and from 6% to 94%, respectively[14,15,17-26].

This happens probably because endoscopic markers cannot be present in milder degrees of the disease. (such as partial villous atrophy) and absent in case of patchy disease[12,18,19]. On the other hand, scalloped feature of duodenal folds has a positive predictive value of 69% for celiac disease and 96% for any duodenal mucosal disease[27]. So, the contradictory evidences and the low sensitivity of endoscopic markers implicates that bioptic sampling should always be performed when the disease is suspected, because their absence does not exclude the diagnosis[16].

WATER-IMMERSION TECHNIQUE

The water-immersion technique is a easy, prompt and safe procedure of enhancement of duodenal villous pattern during a conventional upper endoscopy. Our group developed this technique as a method to emphasize the visualization of duodenal villi[28], and then modified it to make it helpful in clinical practice[29]. The mechanism of the water-immersion technique is very simple, comprising, at first, the removal of air from the duodenal lumen by suction, quickly followed by the injection of 90-150 mL of water[29]. The procedure requests about 25-30 s more than a standard upper endoscopy, resulting very fast. Our group proved the high accuracy of the water-immersion technique in highlighting the duodenal villous pattern in patients undergoing upper endoscopy for the investigation of dyspepsia[29]. This procedure was also trialed in the follow-up of celiac patients after gluten-free diet[30], and also in cases with patchy villous atrophy or villous abnormality limited to the duodenal bulb[11,30], and moreover in children with suspected CD, achieving the same optimal diagnostic accuracy for *in vivo* prediction of areas of the duodenum with villous damage[31]. The water-immersion technique has the potential to reduce the number of biopsy specimens, because of his power of enhancing visualization of areas with villous atrophy (Figure 2A, B); moreover, in patients strongly suspected from CD and with total villous atrophy at water-immersion visualization during upper endoscopy, the high specificity of the procedure could allow to avoid biopsy sampling, with a considerable cost saving[32]. Furthermore, water-immersion technique shows excellent results in terms of operator learning curve, safety, tolerability, and diagnostic accuracy[11,29-32]. In conclusion, for its facility and quickness of performance, and because of its high reliability in evaluating the duodenal villous pattern, the water-immersion technique could potentially be used as a routine procedure during conventional upper gastrointestinal endoscopy, potentially pulling down the number of misdiagnosis of CD, especially when not suspected. Trials with the water-immersion technique has not been replicated by other

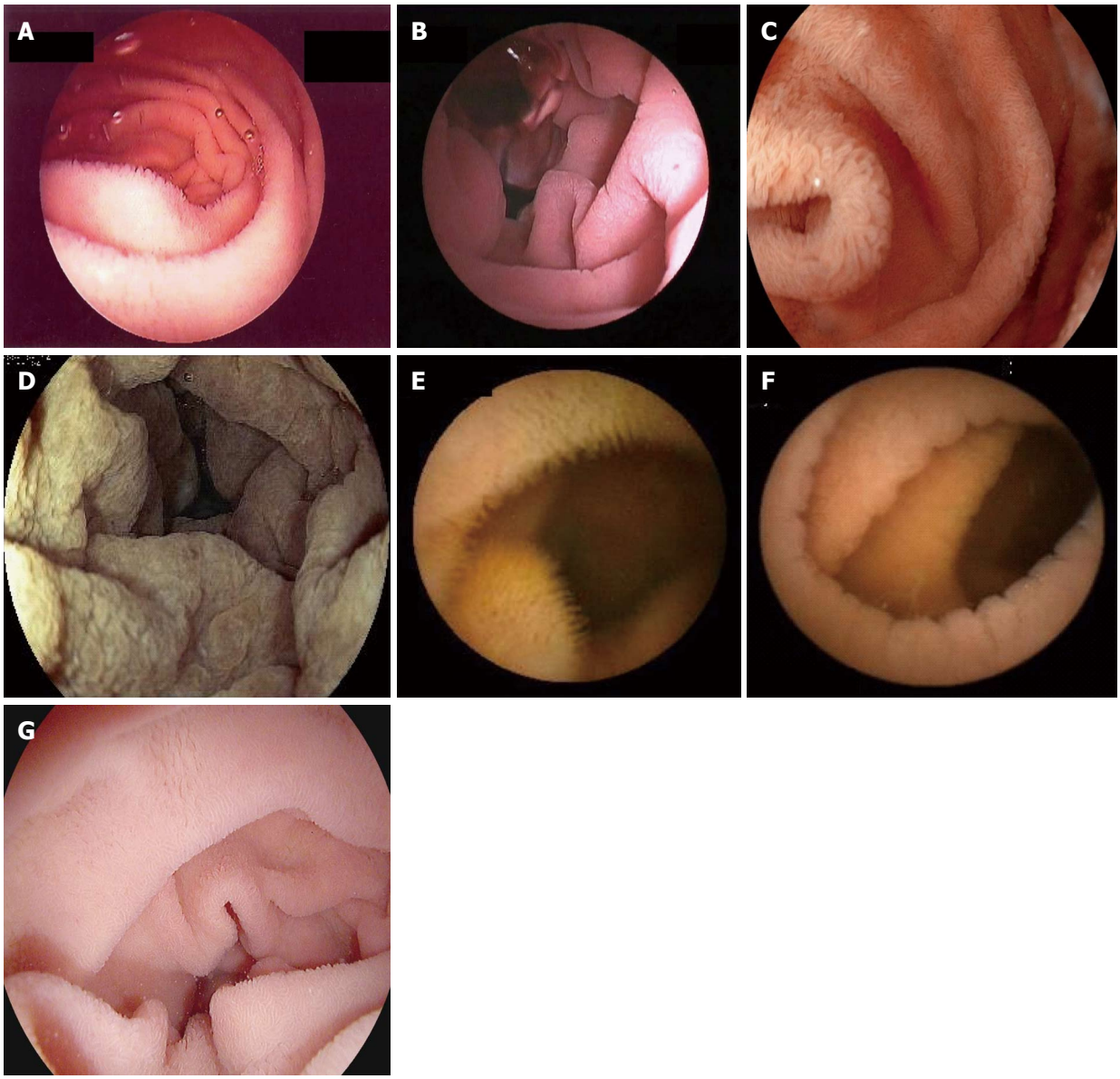

Figure 2 Evaluation of duodenal villous pattern with the water-immersion technique, Fujinon intelligent chromo endoscopy system, capsule endoscopy, I-scan technology. A: Presence of villi with the water-immersion technique; B: Total villous atrophy with the water-immersion technique; C: Presence of villi with Fujinon intelligent chromo endoscopy (FICE) system; D: Total villous atrophy with FICE system; E: Presence of villi with capsule endoscopy; F: Total villous atrophy with capsule endoscopy; G: Duodenal villous pattern with I-scan technology.

groups: therefore, further data, with larger population trials, including large multicenter studies, are required to strengthen this evidence.

CHROMOENDOSCOPY AND HIGH-RESOLUTION MAGNIFICATION ENDOSCOPY

The efficacy of dye-staining chromoendoscopy with indigo carmine or methylene blue in enhancing the visualization of the mucosal surface is nowadays well known[33,34]. The usefulness of chromoendoscopy with indigo carmine for the evaluation of celiac disease was proved yet in 1976[35]. However, this evidence was not confirmed in a latter study[36]. A new generation of endoscopic tools-the "magnification" or "zoom" endoscopes-can produce magnified, high-resolution images (up to 100-135 ×), enhancing details compared to conventional endoscopy[33,37]. They own charged computed device chips with a density of more than 850000 pixels; standard instruments, instead, have charged computer device chips with a density of 100000-300000 pixels. Video endoscopes can provide more and more details about the mucosal surface than conventional ones[38]. The association of indigo carmine-chromoendoscopy and magnification endoscopy in the evaluation of duodenal villous pattern was experienced by Siegel *et al*[39]: this combination showed a sensitivity and specificity of 94% and 88%, respectively for the detection of any villous alteration, and was especially helpful in documenting partial villous atrophy. In a following study, neither this combination technique nor each technology alone showed ad-

vantage compared to standard endoscopy in identifying duodenal lesions such as polyps or hyperplastic Brunner's glands, but anyway authors recognized the role of this combination in case of suspected CD[40]. The role of zoom endoscopy, with a total immersion technique (instillation of 10 mL of water), in the diagnosis of CD was analyzed in 2005[41]: a sensitivity of 90.7%, specificity of 62.9%, a positive predictive value of 83% and a negative predictive value of 77.2% for the diagnosis of any degree of villous atrophy resulted; diagnosis of total villous atrophy was better performed than diagnosis of partial villous atrophy. Cammarota *et al*[42] investigated the combination of magnification endoscopy and water-immersion technique in subjects with suspected duodenal disease, showing a concordance of 100% with histopathology for detecting the absence or the presence of villi. The sensitivity, specificity, positive predictive value and negative predictive value for the detection of total villous atrophy were all 100%, and quite lower for the diagnosis of partial villous atrophy and normal villous patterns. According to other reports, magnification endoscopy could play a role in the detection of patchy villous atrophy[43,44]. In conclusion, enhanced magnification endoscopy, a technique that combines use of acetic acid instillation with magnification endoscopy, has showed a better accuracy in the evaluation of duodenal mucosal pattern than conventional endoscopy[45].

FUJINON INTELLIGENT CHROMO ENDOSCOPY SYSTEM

Fujinon intelligent chromo endoscopy system or optimal band imaging (also known as multiband imaging) is able to assure the same contrast enhancement power of the standard chromoendoscopy, but in a virtual manner. This technology is based on the selection of particular wavelengths from a reflected light signal, resulting in an establishment of digitally created, enhanced images[46]. The usefulness of FICE technology has been successfully proved in Barrett's metaplasia, early gastric cancer, small colorectal tumors[47-49]; moreover, it has showed a great accuracy (100%) for the evaluation of duodenal villi and for the depiction of duodenal villous patterns in CD[50] (Figure 2C, D).

NARROW BAND IMAGING

Narrow-band imaging (NBI) is a new endoscopic technique that allows evaluation of minimal mucosal alterations. NBI uses a narrowed wavelength of light, deriving from the narrowing of the bandwidths of the blue and green filters. This particular wavelength of light is greatly absorbed by hemoglobin, enhancing the visualization of microvascular pattern. It also has a quite deeper superficial penetration than standard white light[51,52]. The efficacy of NBI has been proved in the endoscopic evaluation of a number of diseases, among which also in CD[53,54]. According to Singh *et al*[54], NBI technique is able to detect and grade villous atrophy, with a sensitivity and specificity in detecting villous atrophy of 93.3% and 97.8% respectively, and a sensitivity and specificity in grading villous atrophy of 83.3% and 100%.

OPTICAL COHERENCE TOMOGRAPHY

Optical coherence tomography (OCT) had its debut in medicine in 1991, and nowadays is a cornerstone in ophthalmology, for the usefulness in the evaluation of the retina and atheromasic plaques[55]. The mechanism of OCT is very similar to that of B-mode ultrasonography: OCT detects the echo time delay and the magnitude of back-scattered light waves from various structural tissue features, using interferometry to measure back-scattered light because the delays of reflected light are too little for a direct electronic measurement[55-57]. The images performed by OCT resemble those generated by B-mode ultrasound and endoscopic ultrasonography; however, the resolution of OCT is better (5-10 mm)-because of the use of light instead of sound waves-, closer to the histological images[55,56,58]. So, OCT allows the study of the proximal layers of gastrointestinal (GI) wall, and may be helpful in the early diagnosis of neoplasms[57]. The usefulness of OCT has been proved yet in the study of GI malignancies[59,60], Barrett's esophagus and dysplasia[61-67], pancreatic and biliary ducts[68,69], and other diseases. Preliminary reports from Masci *et al*[70-72], the use of OCT *in vivo* during real-time endoscopic imaging generated promising results for the evaluation of duodenal villous morphology. These authors, in fact, found total concordance between OCT and histology results for the evaluation of villous morphology in both patients with CD and healthy individuals, also in children, exactly identifying, furthermore, different degrees of villous atrophy.

CONFOCAL LASER ENDOMICROSCOPY

Confocal laser endomicroscopy, or confocal endomicroscopy, is a novel technology that allows an *in-vivo* microscopy of the human gastrointestinal mucosa during upper or lower endoscopy[73,74]. Endomicroscopy has been applied in a number of gastrointestinal diseases, and also in CD[73-77]. In particular, in the experience of Zambelli *et al*[76], the images obtained by confocal endomicroscopy and histology were similar, both for negative subjects and for celiac patients; moreover, in celiac patients confocal endomicroscopy was able to identify moderate-to-severe villous atrophy, but quite less to visualize the crypt hyperplasia and flogistic infiltration. In a case report, CD was diagnosed *in vivo* by confocal endomicroscopy on the basis of the presence of complete villous atrophy and a rise of intraepithelial lymphocytes[77].

VIDEOCAPSULE ENDOSCOPY

Capsule endoscopy is a useful, patient-friendly method for the evaluation of the whole small bowel. Obscure

gastrointestinal bleeding is the strongest indication for capsule endoscopy[78]; however, recent evidences point out new, intriguing purposes and indications: in particular, regarding the object of this review, the role of capsule endoscopy in the diagnosis and follow-up of CD is growing up quickly[79-91]. The optical system of the capsule possesses a 8-folds magnification power, that allows to easily evaluate the duodenal villous pattern (Figure 2E, F). Moreover, it allows an evaluation of the small intestine along its whole length. Capsule endoscopy seems to be able to recognize the endoscopic markers of celiac disease described in the literature, such as scalloping and reduction in number of duodenal plicae, nodularity and mosaic pattern of mucosa[81,82,86,87].

In an initial multicenter trial, capsule endoscopy had an excellent reported sensitivity and specificity of 87.5% and 90.9%, respectively, for the detection of villous atrophy as compared with the criterion standard of duodenal histology[84], but such promising data have not been confirmed in the series presented by the same group[85]. Summarizing the most important studies about the role of capsule endoscopy in CD, it counts a high sensitivity (range, 70%-95.2%), a quite less high specificity (range, 63.6%-100%) and high positive predictive value (96.5%-100%), but a lower negative predictive value (71.4%-88.9%)[82,83,85,88]. These results are cheerful, but the relatively low negative predictive value indicates that CD can't be surely excluded by a negative evaluation at capsule endoscopy.

It should be noted that there is not an overall high degree of agreement between investigators (range 0.41-0.87), and it probably denotes a difficulty in evaluating correctly villous atrophy even if operators are well-experienced in video capsule enteroscopy.

However, the use of capsule endoscopy could be considered in patients with positive tissue transglutaminase or anti-endomysial antibodies who are unable or unwilling to perform an upper endoscopy[89], and also for the evaluation of the whole small bowel in patients with positive antibodies and duodenal histology negative for CD, even if regarding evidences don't confirm this hypothesis[90]. More realistically, capsule endoscopy can be very useful in case of suspected refractory or complicated CD. In particular, capsule endoscopy can detect alterations such as malignancy or ulcerative jejunitis in refractory celiac disease (RCD) type Ⅱ, but evidences are not so bright regarding RCD type Ⅰ [91].

I-SCAN TECHNOLOGY

I-scan technology is an image enhanced endoscopy technology recently developed by Pentax Medical®, Japan[92]. It can be classified among digital contrast methods. It allows three different modalities of image enhancement: surface enhancement (SE), contrast enhancement (CE), and tone enhancement (TE). SE enhances light-dark contrast by obtaining luminance intensity data for each pixel. CE digitally adds blue color in relatively dark areas, enhancing minute irregularities on the mucosal depressed areas. Both enhancement functions work in real time without impairing the original color of the organ. TE separates and analyzes the individual red, green and blue components of a normal image; the algorithm then alters the color frequencies of each component, recombining the components to a single, new color image. For SE and CE, it is possible to switch among three enhancement levels (low, medium and high). At now, three types of TE are available: TE-e (for esophagus), TE-g (for stomach) and TE-c (for intestine). Switching the levels or modes of enhancements can be done on a real-time basis, without any time lag, by pushing a relevant button.

I-scan technology has been applied to several field of interest in gastrointestinal endoscopy, such as colorectal lesions[93-97], Whipple's disease[98], gastroesophageal reflux disease[99-101], Barrett's esophagus[102]. Recently, our group has experienced I-scan technology for the evaluation of duodenal villous pattern[103], with the following results: I-scan system was demonstrated to have great accuracy (100%) in the detection of marked villous atrophy patterns and quite lower accuracy in determining partial villous atrophy or normal villous patterns (respectively, 90% for both items) (Figure 2G).

Therefore, I-scan technology seems to be a reliable tool also for the diagnosis of CD. Obviously, further, larger studies are needed to confirm this feeling.

CONCLUSION

The recent advances in terms of technology and techniques of endoscopy, reviewed above, can certainly improve our diagnostic possibilities in the evaluation of CD, and should not be ignored, but accepted with wisdom. Surely, it is important to perform these tools in appropriate endoscopic centers, owning good equipment and enough expertise. Moreover, in a hypothetic world without biopsy sampling, but with a virtual histological analysis, a gastroenterologist can not absolutely brush aside a solid histological training. Therefore the most realistic scenario is not a replacement, but an interaction between endoscopic and histological analysis: a similar "joint-venture" might knock down misdiagnoses and reduce overall costs of diagnostic course of CD: large, randomized trials, also with cost analyses and clinical outcome evaluations, are needed to carry out this concept.

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REVIEW

Oral manifestation in inflammatory bowel disease: A review

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Abstract

Inflammatory bowel diseases (IBDs), including Crohn's disease (CD) and ulcerative colitis, not only affect the intestinal tract but also have an extraintestinal involvement within the oral cavity. These oral manifestations may assist in the diagnosis and the monitoring of disease activity, whilst ignoring them may lead to an inaccurate diagnosis and useless and expensive workups. Indurated tag-like lesions, cobblestoning, and mucogingivitis are the most common specific oral findings encountered in CD cases. Aphthous stomatitis and pyostomatitis vegetans are among non-specific oral manifestations of IBD. In differential diagnosis, side effects of drugs, infections, nutritional deficiencies, and other inflammatory conditions should also be considered. Treatment usually involves managing the underlying intestinal disease. In severe cases with local symptoms, topical and/or systemic steroids and immunosuppressive drugs might be used.

Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Extra-intestinal manifestations; Pyostomatitis vegetans; Aphthous stomatitis; Cobblestoning; Mucogingivitis; Oral manifestation

Core tip: Although the gastrointestinal tract is the primary site of involvement in inflammatory bowel disease (IBD) patients, some cases might present with non-intestinal manifestations, such as oral lesions. These oral manifestations may aid in the diagnosis and the monitoring of disease activity, whilst ignoring them may lead to an inaccurate diagnosis and useless and expensive workups. Indurated tag-like lesions, cobblestoning, mucogingivitis, aphthous stomatitis, and pyostomatitis vegetans are the main oral presentations of IBDs. With the growing incidence of IBDs and the increased likelihood of encountering these particular manifestations, this review summarizes various oral findings seen in IBD cases by describing their unique morphologic description, treatment recommendations, and probable differential diagnosis.

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INTRODUCTION

Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are chronic inflammatory diseases with primary intestinal involvement[1-5]. Although the exact underlying pathogenesis of IBD has not been clearly elucidated, it is postulated that dysregulated immunity is its basis[4,6-12]. Generally, it is assumed that IBD is a multifactorial disease in which immune system, genetics, and environmental factors all have a

role[2,8,13-17]. Other than the expected symptoms of gastrointestinal involvement, IBD patients may exhibit a wide range of non-intestinal signs and symptoms known as extraintestinal manifestations (EIMs), with prevalence rates ranging from 6%-47%[2,8,18,19]. Approximately one third of IBD patients develop EIMs in the course of their disease[1,20-25]. Joints, skin, eyes, and the biliary tract are among the most common organs involved in EIMs[22,26-28]. Oral involvement with different presentations may also be seen in IBD. Oral manifestations could also occur in these patients due to other causes, such as drug reactions, infections, and unrelated diseases[1,2,6,8,20,21]. Patients with IBD may present with these oral manifestations years before the appearance of intestinal disease[1,6]. Recognizing these patterns may assist physicians and other care givers in making a timely diagnosis of IBD while avoiding unnecessary workups[29]. The scope of this review is to describe various oral presentations in IBD and their differential diagnosis and treatment.

EPIDEMIOLOGY OF ORAL MANIFESTATIONS IN IBD

In 1969, Dyes and colleagues initially described oral lesions in two patients with CD[7,30]. This was followed in the same year by Dudeney's report of another patient suffering from CD who had oral manifestation[31]. Oral lesions in the absence of intestinal findings in CD were initially described in 1972 by Varley[32], and there have since been various reports on the incidence of oral lesions in CD[1-3,23,30,33-39]. The highest rate was reported from a study in a pediatric age group, indicating a rate of 48%[33]. The prevalence rate is estimated to be between 50% and 20% in most publications[1,33,38].

This variation in rate might be related to the different ages of patients studied, their ethnicity and genetic background, whether they were receiving treatment while being investigated, the experience level of the examiners, and the variation in definition of specific lesions[34].

In the majority of cases, intestinal involvement precedes the oral lesion[1]. Oral lesions are more common in CD compared to UC, more prevalent in children compared to adults, and with a male dominance[1,8,21,33,34,37,40,41]. The prevalence is also higher in CD patients with proximal gastrointestinal tract and/or perianal involvement[2,33,42].

Oral lesions may be the primary presenting signs preceding gastrointestinal symptoms[43,44] in 5%-10% of affected patients[39]. This figure has been reported to be as high as 60% in patients with CD[37].

Although the lesions might be more severe at the time of active disease, the correlation is not universal, and up to 30% of affected patients may continue to manifest oral lesions (especially in the pediatric age group) despite disease control[34,45].

ORAL LESIONS IN CROHN'S DISEASE

Dudeney's report of oral Crohn's disease in 1969 described it as a raised, edematous, pink granulation tissue in the buccal mucosa[31]. It is now known that the lips are the most frequent site of oral Crohn's disease (OCD) lesions[37]. Oral lesions may be painful, impair proper oral function, or lead to psychological disorders due to disfigurement[8,46]. Oral manifestations of CD can be specific or non-specific, based on the presence of granulomas noted on the histopathology reports[1].

SPECIFIC ORAL CROHN'S DISEASE LESIONS

These specific lesions contain granulomatous changes noted upon histopathological examination. They are less common than non-specific lesions, and can occur either concomitantly with intestinal symptoms or before gut presentation by several years[47,48]. The most affected portions in the mouth are the buccal mucosa, gingiva, lips, vestibular, and retromolar areas[32]. There are four main lesions, as described below and shown in Table 1.

INDURATED TAG-LIKE LESIONS

These are white reticular tags[35] referred to as mucosal tags, epithelial tags, or folds[49]. These lesions are mostly discovered in the labial and buccal vestibules, and in the retromolar regions[21]. Up to 75% of these lesions may show non-caseating granulomas on histopathology[33,42]. There has been no specific direct association of these lesions with intestinal CD activity reported[1]. Treatment is described in the later section on general treatments of OCD lesions.

COBBLESTONING

Fissured swollen buccal mucosa with corrugation and hyperplastic appearance of the mucosa are called cobblestoning[1,42,50,51]. These lesions are usually seen in the posterior buccal mucosa and may be associated with succulent mucosal folds with normal epithelium[21]. The lesions usually consist of mucosal-colored papules that produce firm plaques on the buccal mucosa and palate. Such lesions may cause pain and make speaking and eating normally difficult[52]. These lesions, along with mucosal tags, are considered pathognomonic for CD[35], but are not associated with intestinal CD activity[1]. Treatment consists of topical steroids in addition to the treatment of intestinal involvement. In more severe presentations, systemic steroids could be used[53].

MUCOGINGIVITIS

The gingiva may become edematous, granular, and hyperplastic in Crohn's disease, with or without ulceration. The whole gingiva up to the mucogingival line might be involved[7,30]. As with other specific lesions of the oral cavity, this lesion has no association with intestinal CD activity. Treatment is discussed in the section on general

Table 1 Summary of specific and non-specific oral lesions in Crohn's disease

	Lesion	Relation with CD activity	Frequency	Treatment options
Specific oral lesions	Indurated tag-like lesions	No specific direct association reported	Common in OCD patients	See general points on the treatment of OCD in the text
	Cobblestoning	No specific direct association reported	Common in OCD patients	Topical steroids for less severe cases and systemic steroids for others
	Mucogingivitis	No specific direct association reported	Common in OCD patients	See general points on the treatment of OCD in the text
	Others:			
	Lip swelling with vertical fissures	No specific direct association reported		Topical tacrolimus, intra-lesional injection of steroids, immunosuppressive agents
	Deep linear ulcerations			Topical analgesics, 5-ASA, or steroids, intra-lesional steroids, topical tacrolimus, other medications used in PV treatment
Non-specific oral lesions	Aphthous stomatitis	No specific direct association reported	10% of patients with UC and 20%-30% of those with CD	Topical agents (lidocaine 2%, triamcinolone 0.1%, dexamethasone elixir), non-steroidal anti-inflammatory pastes, systemic steroids, intra-lesional steroids
	Pyostomatitis vegetans	Associated with active CD	Rare	Antiseptic mouthwashes/topical steroids (though less effective), systemic steroids, azathioprine and sulfamethoxypyridazine, dapsone, cyclosporine A, injections of infliximab pursued by maintenance therapy with MTX, adalimumab, surgical colectomy in UC
	Others:			
	Angular cheilitis	No specific direct association reported	Unknown	5-ASA mouthwashes, topical steroids (1% hydrocortisone), vitamin supplements, intra-lesional steroids
	Persistent submandibular lymphadenopathy			See general points on the treatment of OCD in the text
	Recurrent buccal abscesses			Antibiotics, infliximab, methotrexate, thalidomide
	Perioral erythema with scaling			
	Glossitis			

CD: Crohn's disease; OCD: Oral Crohn's disease; MTX: Methotrexate; UC: Ulcerative colitis.

treatments of OCD lesions below.

OTHER SPECIFIC LESIONS

Lip swelling with vertical fissures, deep linear ulcerations (usually in the buccal sulci with hyperplastic folds), and midline lip fissuring may also occur in CD[1,2,7,8,22,30,33,35,39,42,49,54]. These lesions also have no association with intestinal CD activity[1].

While these lesions may be very incommodious for patients, they can be treated with topical tacrolimus at low concentration (0.5 mg/kg) and intra-lesional steroid injection with or without mandibular blockade[34,55,56]. In more severe cases with persistent pain and cosmetic disfigurement, more aggressive therapy with immunosuppressive agents is recommended[34].

NON-SPECIFIC ORAL LESIONS IN CD

Table 1 provides details of various non-specific oral lesions that occur with Crohn's disease.

APHTHOUS STOMATITIS

Aphthae are shallow round ulcerations with central fibrinous exudate and an erythematous border[23,57]. These lesions may occur in 20%-25% of the general population[3,58], up to 10% of patients with UC, and 20%-30% of those with CD that have oral aphthosis[4]. In a survey conducted in Iran, oral aphthous lesions were found in approximately 13% of CD *vs* 6% of UC patients[13]. The association of oral aphthosis and disease activity is not clear. While it may become more severe in active disease, its presence does not correlate with disease activity.

Patients with IBD and other EIMs may suffer recurrent aphthous stomatitis more often than others[4]. Aphthous stomatitis has been associated with ankylosing spondylitis, uveitis, peripheral arthritis, and erythema nodosum[59]. Aphthous eruptions are not specific for IBD and may be observed in several other disorders including celiac sprue, HIV/AIDS, Behçet's disease, and Reiter's syndrome, as well as common aphthae seen in the normal population[23,60-66].

Management of CD is usually sufficient for control of oral aphthosis. For control of pain, topical agents (such as lidocaine) and/or topical steroids (such as triamcinolone 0.1%) up to three times per day can be used. Dexamethasone elixir (0.5 mg/5 mL spit or swish) or ointment up to three times per day is also efficacious. Non-steroidal anti-inflammatory pastes are effective in

relieving pain and promoting healing. Systemic or intra-lesional steroids should be reserved for severe refractory and/or persistent cases[4,13,21,32,67-70].

PYOSTOMATITIS VEGETANS AND OTHER NON-SPECIFIC LESIONS

Pyostomatitis vegetans can occur in both UC and CD, but is more common in the former and will be discussed in more detail in the later section addressing oral manifestations of UC.

Other non-specific oral findings of CD include angular cheilitis, persistent submandibular lymphadenopathy, sicca syndrome and reduced salivation, halitosis, dental caries and periodontal involvement, candidiasis, odynophagia, dysphagia, minor salivary gland enlargement, perioral erythema with scaling, recurrent buccal abscesses, glossitis, mucosal discoloration, lichen planus, and metallic dysgeusia[2,7,21,32,34,35,40,54,71]. For the management of angular cheilitis, 5-ASA mouthwashes, topical steroids (1% hydrocortisone), vitamin supplements, and intra-lesional steroids may be effective. Antibiotics are the first step in treating recurrent buccal abscesses. For more severe cases, immunomodulating agents including chimeric anti-tissue necrosis factor (TNF) alpha monoclonal antibody-infliximab, methotrexate, and thalidomide have been used[7,21].

GENERAL POINTS ON THE TREATMENT OF OCD

In the majority of patients with OCD, the oral findings are asymptomatic and clinically silent. In these patients, no peculiar treatment is needed for oral lesions and the latter will resolve over time in association with the treatment of gastrointestinal disease using anti-inflammatory drugs (5-ASA), immunosuppressive agents, and finally biological agents, whichever are indicated[8,21,34,40,72].

The treatment armamentarium includes topical and systemic steroids, 5-ASA compounds, immunosuppressive agents, biologic treatments, and even antibiotics such as tetracycline[2,73].

The first and foremost step in treating oral lesions is to control colonic disease[74]. Food restriction, which is discussed later in the management of orofacial granulomatosis (OFG), could also be tried in OCD[75,76].

ORAL LESIONS IN UC

There are many similarities between the oral manifestations of CD and UC. Although oral lesions are more common in CD, almost all of the so-called non-specific oral lesions described in CD can also occur in UC. Among these lesions, pyostomatitis vegetans occurs more commonly in UC than in CD and will be discussed here in more detail[1,2,74,77,78].

The term pyostomatitis vegetans (PV) was first introduced by McCarthy in 1949[38], but its association with IBD was not initially recognized[38]. PV is a chronic mucocutaneous ulcerative disorder consisting of multiple miliary white or yellow pustules with an erythematous and edematous mucosal base[1,23,77,79]. The pustules can rupture and coalesce to form linear or "snail-track" ulcers[1,23,38,77,78,80]. The most frequently involved regions of the oral cavity are the labial gingiva, labial, and buccal mucosa[78]. The least damaged portions are the tongue and floor of the mouth[1], but pustules can involve almost all parts of the oral cavity[78].

PV can be seen at any age, but is more prevalent in patients aged between 20 and 59 years, with an average age of 34 years. Males are affected more frequently than females, with a ratio of 2:1-3:1[81,82]. PV is considered to be the oral equivalent of pyodermatitis vegetans on the skin[77,78]. There is a strong association between PV and IBD, and the former is a specific marker of disease activity in UC[1,2,38,39,78,83,84]. Intestinal involvement usually predates the onset of PV in IBD, although this could be asymptomatic and mild[23,85]. Despite every effort, no bacterial, fungal, or viral cause has yet been found for this manifestation and its pathogenesis remains obscure[77]. The principal histological features on microscopy are intra-epithelial and/or sub-epithelial micro-abscesses with neutrophils and eosinophils. Furthermore, hyperkeratosis, acanthosis, and acantholysis are seen in histology examination[1,38,40,78,86]. Direct immunofluorescence in PV is negative for deposits of IgA, IgG and C3 and this result is helpful in distinguishing PV from pemphigus vulgaris[1,87]. Clinically, the patient may have fever, enlarged and tender submandibular lymph nodes, and pain. Pain intensity is variable; some patients with extensive oral lesions may not have any pain[78]. Peripheral eosinophilia is seen in up to 90% of cases and is the main laboratory finding in many patients[87].

The diagnosis of PV is based on a constellation of clinical features that include concurrent IBD, peripheral eosinophilia, histological study, and negative culture results of the lesion exudate. As mentioned above, a negative immunofluorescence study is also helpful[1,77,78].

The main differential diagnoses of PV include vesicular disorders involving both the skin and oral cavity; similar to pemphigus vulgaris in particular, as well as other diseases like bullous pemphigoid, acquired epidermolysis bullosa, bullous drug eruptions, herpetic infection, Behçet's disease, and erythema multiforme[1,77,80,88]. Herpetic infections should be excluded by Tzanck smear, antigen detection, and culture of the virus, or PCR for HSV virus[23]. The mainstay in the management of PV is the treatment of underlying IBD. Topical steroids and antiseptic mouthwashes alone are effective in only a few instances. Systemic steroids are usually prescribed for these patients and are considered as the treatment of choice. Azathioprine and sulfamethoxypyridazine can be used in parallel with steroids as sparing agents[3,21,23,38,77,78]. Dapsone is another option, but should be used as a second line agent, especially in relapsing cases. Hemolytic ane-

mia, hepatitis, agranulocytosis, and drug-induced allergic reactions are the major side effects of dapsone requiring attention[3,78].

Calcineurin blockers like cyclosporine A have been successfully used, as described in case reports in the treatment of PV[89]. Injections of infliximab followed by maintenance therapy with methotrexate have been also effective, especially in PV associated with CD[77]. Systemic use of newer humanized anti-TNF agents like adalimumab has also proven effective in inducing remission of both oral and gastrointestinal manifestations[77]. Surgical colectomy produces promising results in PV associated with UC[3,78,90].

Other non-specific findings in UC include oral aphthae, glossitis, cheilitis, stomatitis, lichen planus, mucosal ulcers, diffuse pustules, and non-specific gingivitis[1-3,23,42].

In a report of patients with UC, 4.3% had aphthous stomatitis during intestinal disease flare-ups[2], thus the presence of this non-specific manifestation may have some correlation with disease activity in UC.

DIFFERENTIAL DIAGNOSES

Because CD is a granulomatous disorder, all other diseases capable of inducing granulomatous reaction in the oral cavity are included in the differential diagnosis (DDX) list. The most common cause of developing oral granulomas is a response to foreign bodies, primarily dental materials such as retained amalgams or endodontic sealers[91]. The second important DDX to be considered is tuberculosis bacilli. Special staining processes for acid-fast bacilli, PPD skin test, sputum culture, and chest X-ray are often used to diagnose oral tuberculosis[2,80,92].

Fungal infections such as candidiasis, histoplasmosis, cryptococcosis, paracoccidioidomycosis, and blastomycosis can all trigger granulomatous involvement of the mouth. The presence of these infections could be confirmed by special stains including applying PAS or Gömöri trichrome stain and, more specifically, with molecular studies[2,21,80].

Oral sarcoidosis should always be considered in DDX, and an appropriate workup should include measuring serum angiotensin converting enzyme, IL-2 receptor level, IL-8 level, and chest X-ray in suspected cases[2,6,21,39,93].

Leprosy, cat scratch disease, tertiary syphilis, orofacial granulomatosis, T-cell lymphoma, and Wegener's disease can all produce a granulomatous reaction in the oral cavity, but are much rarer and usually have other prominent features leading to diagnosis[21,39].

Considering the role of nutritional deficiencies is of utmost importance as stomatitis, glossitis, aphthous ulcers, cheilitis, or perioral dermatitis may occur with nutrient deficiencies resulting from an insufficient supply of the vitamin B family, albumin, iron, folate, zinc, niacin, and/or other essential elements[8,41,94-97]. Nutrient deficiencies may be the result of intestinal involvement or may be caused by the medications used in the treatment of IBD[98,99]. Sulfasalazine and azathioprine, for instance, may cause folate and niacin deficiency, respectively[2].

Other non-specific oral manifestations may also be related to the side effects of drugs. As an example, oral aphthosis has been reported in association with non-steroidal anti-inflammatory agents, nicorandil[100], and bupropion[101]; gingival hyperplasia with cyclosporine[102], amlodipine[103], and anticonvulsants such as phenytoin[104]; and reversible lichen planus with sulfasalazine[54].

OROFACIAL GRANULOMATOSIS

Gibson *et al*[40] used the term OFG in 1985 to define a constellation of oral signs similar to those seen in OCD, but in the absence of evidence of intestinal CD. In this rare syndrome, chronic swelling of the lips and lower half of the face is prominent, in association with oral ulcers and hyperplastic gingivitis. Granulomatous cheilitis is the most common sign seen in OFG[105]. The most frequent sites of involvement in OFG are the lips, which may be individually or both involved[80]. Lip swelling usually leads to painful vertical fissures[2]. Three forms of ulcers are found in OFG: deep buccal ulcers with raised peripheral mucosa, aphthous-type ulcers, and micro-abscesses located commonly on the gingival margin or soft palate[21]. In general, the ulcers are mainly superficial and the gingivae are erythematous with patchy distribution, mostly affecting the anterior portion. These alterations extend from the free gingival margin to the non-keratinized mucosa of the sulci, developing a full-thickness gingivitis pattern[40].

In the largest series of studies involving OFG reported to date, the mean age of those affected at presentation was 20 years with no gender predilection. With the pathogenesis unknown, allergic, infectious, and genetic causes have also been postulated[40,106]. Unlike OCD in which Th1 $CD4^+$ lymphocytes are the dominant population, in OFG the overstimulation of Th2 $CD4^+$ lymphocytes is detected in biopsy specimens, where it is shown as infiltrating cells[21].

Granulomas noted upon histology examination are the hallmark in both OFG and OCD. The only way to exclude CD is by clinical presentation[21]. As mentioned previously, oral manifestations may precede gastrointestinal involvement in CD for many years. Thus, cases labeled as OFG may later progress to being diagnosed as CD[21,34]. Recently, it has been reported that 4 out of 6 children with OFG in early childhood were reported as having developed CD on follow-up[34].

A rare presentation of OFG seen in adults is Melkersson-Rosenthal syndrome; a triad of orofacial swelling, intermittent facial paralysis, and a fissured tongue[21,34,107].

Observational studies in pediatric patients with OFG have demonstrated that dietary elimination of some triggering elements (encompassing cinnamaldehyde, benzoate additives, carnosine, monosodium glutamate, cocoa, and sunset yellow) are effective in the treatment of oral lesions[75,76]. Analgesia and topical agents like beclomethasone mouthwash and 5-ASA spray or ointments can be

used as basic therapies. In unresponsive cases, treatment with systemic steroids and immunosuppressive medications can be used[21]. Clofazimine, a drug used in the treatment of leprosy, is occasionally effective in OFG[37].

CONCLUSION

Oral manifestations of inflammatory bowel diseases are diverse. Although they are generally more common in patients with Crohn's disease, specific manifestations like PV occur more commonly in ulcerative colitis, which is associated with disease activity in most instances. Most other manifestations have no correlation with disease activity. In differential diagnosis of these oral manifestations, side effects of drugs, nutritional deficiencies, infections, as well as other granulomatous diseases with oral involvement should all be considered. There is usually no need for specific treatment for these lesions, but when indicated it may comprise topical and systemic steroids, immunosuppressive drugs, antibiotics, and even biological treatment in more severe cases.

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REVIEW

Endoscopic papillary large balloon dilation for the removal of bile duct stones

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Abstract

Endoscopic papillary large balloon dilation (EPLBD) with endoscopic sphincterotomy (EST) has been widely used as the alternative to EST along with endoscopic mechanical lithotripsy (EML) for the removal of large or difficult bile duct stones. Furthermore, EPLBD without EST was recently introduced as its simplified alternative technique. Thus, we systematically searched PubMed, Medline, the Cochrane Library and EMBASE, and analyzed all gathered data of EPLBD with and without EST, respectively, by using a single standardized definition, reviewing relevant literatures, published between 2003 and June 2013, where it was performed with large-diameter balloons (12-20 mm). The outcomes, including the initial success rate, the rate of needs for EML, and the overall success rate, and adverse events were assessed in each and compared between both of two procedures: "EPLBD with EST" and "EPLBD without EST". A total of 2511 procedures from 30 published articles were included in EPLBD with EST, while a total of 413 procedures from 3 published articles were included in EPLBD without EST. In the results of outcomes, the overall success rate was 96.5% in EPLBD with EST and 97.2% in EPLBD without EST, showing no significant difference between both of them. The initial success rate (84.0% *vs* 76.2%, $P < 0.001$) and the success rate of EPLBD without EML (83.2% *vs* 76.7%, $P = 0.001$) was significantly higher, while the rate of use of EML was significantly lower (14.1% *vs* 21.6%, $P < 0.001$), in EPLBD with EST. The rate of overall adverse events, pancreatitis, bleeding, perforation, other adverse events, surgery for adverse events, and fatal adverse events were 8.3%, 2.4%, 3.6%, 0.6%, 1.7%, 0.2% and 0.2% in EPLBD with EST and 7.0%, 3.9%, 1.9%, 0.5%, 0.7%, 0% and 0% in EPLBD without EST, respectively, showing no significant difference between both of them. In conclusion, recent accumulated results of EPLBD with or even without EST suggest that it is a safe and effective procedure for the removal of large or difficult bile duct stones without any additional risk of severe adverse events, when performed under appropriate guidelines.

Key words: Balloon dilation; Endoscopic sphincterotomy; Common bile duct gallstones; Lithotripsy; Complications; Assessment; Patient outcomes

Core tip: We systematically analyzed all gathered data of endoscopic papillary large balloon dilation (EPLBD) with and without endoscopic sphincterotomy (EST), respectively, by using a single standardized definition, to evaluate their outcomes, reviewing relevant literatures. Thirty studies involving 2511 procedures of EPLBD with EST and 3 studies involving 413 procedures of EPLBD without EST were enrolled in this review. The results of EPLBD with or even without EST suggest that it is a safe and effective procedure for the removal of large or difficult bile duct stones without any additional risk of severe adverse events, when performed under appro-

priate guidelines.

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INTRODUCTION

Ever since its introduction in 1974[1,2], endoscopic sphincterotomy (EST) has become the standard procedure for the removal of common bile duct stones. However, it still runs the risk of various adverse events, such as bleeding, perforation, pancreatitis and cholangitis[3-6], and large bile duct stones may require endoscopic mechanical lithotripsy (EML) as an adjunctive procedure to facilitate stone clearance[7-11]. Endoscopic papillary balloon dilation (EPBD) was first proposed as an alternative to EST in 1982[12]. Initially it was widely performed in the belief that it had more advantages over EST such as the reduction of bleeding and perforation risks and functional preservation of the biliary sphincter[13-17]. However, it has been proven that EPBD is significantly less successful in removing bile duct stones compared to EST, because dilating balloons with a range of 6- to 10-mm in diameter are inadequate in achieving an ampullary opening wide enough[18,19]. More importantly the risk of pancreatitis is significantly higher than EST to the extent of an increased mortality rate[7,18,20].

Endoscopic papillary large balloon dilation (EPLBD) combined with EST was initially introduced to facilitate in the removal of large bile duct stones in 2003[21], where large-diameter balloons (12- to 20-mm balloon) are used to remove large or difficult bile duct stones[22-26]. It was initially presumed that this new technique would cause higher incidence rates of potential serious adverse events such as pancreatitis and bile duct perforation[27-31]. However, recent results on EPLBD with EST have quashed these presumptions[32-36], therefore it is rapidly and widely being adopted as a useful technique for the removal of large or difficult bile duct stones[37-50]. As an alternative technique, EPLBD without EST was formally incorporated as a simplified technique in 2009[51]. A number of studies have recently been conducted in South Korea and Taiwan[43,45,52,53], concurring that it is also as safe and effective in patients with large bile duct stones without any additional risk of severe pancreatitis or perforation. Nevertheless, it was very difficult to get a precise analysis of the outcomes of EPLBD, because the results from each article were based on different definitions. Thus, we analyzed all gathered data of EPLBD with and without EST, respectively, by using a single standardized definition, reviewing relevant literatures.

LITERATURE SEARCH AND REVIEW

A search of literatures on EPLBD was initially performed under title and abstract with the search terms "large balloon", "balloon dilation", "sphincteroplasty" and "endoscopic papillary large balloon dilation" by means of the commonly used online databases; PubMed, Medline, the Cochrane Library and EMBASE. After reviewing the corresponding abstracts of the retrieved articles, those that showed relevance to this review were downloaded in full text. Additional articles were then searched by tracing back on their references. Details of literature search and evaluation process are shown in Figure 1.

The following inclusion criteria were employed in this review: (1) original articles about clinical trials in humans published between 2003 and 2013 June, since EPLBD was first reported on in 2003[21]; (2) the language filtering system was not used in online databases; (3) EPLBD performed with large-diameter balloons (12-20 mm) whether preceding EST was done or not; and (4) EPLBD performed when the standard balloon and basket techniques after EST failed even though the stone size was under 10 mm. Exclusion criteria of patients or articles were as follows: (1) review articles[54-58], editorial letters[59-63], case reports[64-68], case series[69] and preliminary reports[70]; (2) articles which included EPBD with a dilating balloon less than 12 mm in diameter[52,53,71-76]; (3) articles about clinical trials on only patients with surgically altered anatomy of the upper gastrointestinal tract, such as Billroth II surgery and Roux-en-Y anastomosis[77-82]; (4) articles where data extraction were not possible[83-86]; and (5) articles which contained duplicated patient data from another publication[87].

Patient data from the relevant articles was independently extracted by two reviewers and is as follows; baseline clinical characteristics of the patients, study design, study inclusion criteria, a history of gastrointestinal surgery, periampullary diverticulum, largest stone size, range of stones, number of stones, treatment naïve, performance of EST, size of EST, prior EST, balloon diameter, time duration of inflated balloon, initial success rate, success rate of EPLBD without EML, rate of use of EML, overall success rate, number of sessions needed for complete stone removal, rates of adverse events and rate of surgery and mortality due to adverse events. An article of a large scaled multicenter study[43], that included our institute, where the data of both the patients who had EPLBD with EST and those without EST were calculated as one, was re-analyzed using its raw data in order to re-group both of them separately. Any discrepancies between the two reviewers' results were resolved through discussion.

DEFINITION

Because data from each article, such as size of EST, initial success rate, success rate of EPLBD without EML, rate of use of EML, overall success rate, and rate of adverse events, was based on different definitions, we re-analyzed all gathered data by using a single standardized definition, in order to get a precise analysis of the outcomes. The size of the EST used before performing EPLBD was classified into 2 groups based on the extent of ampullary

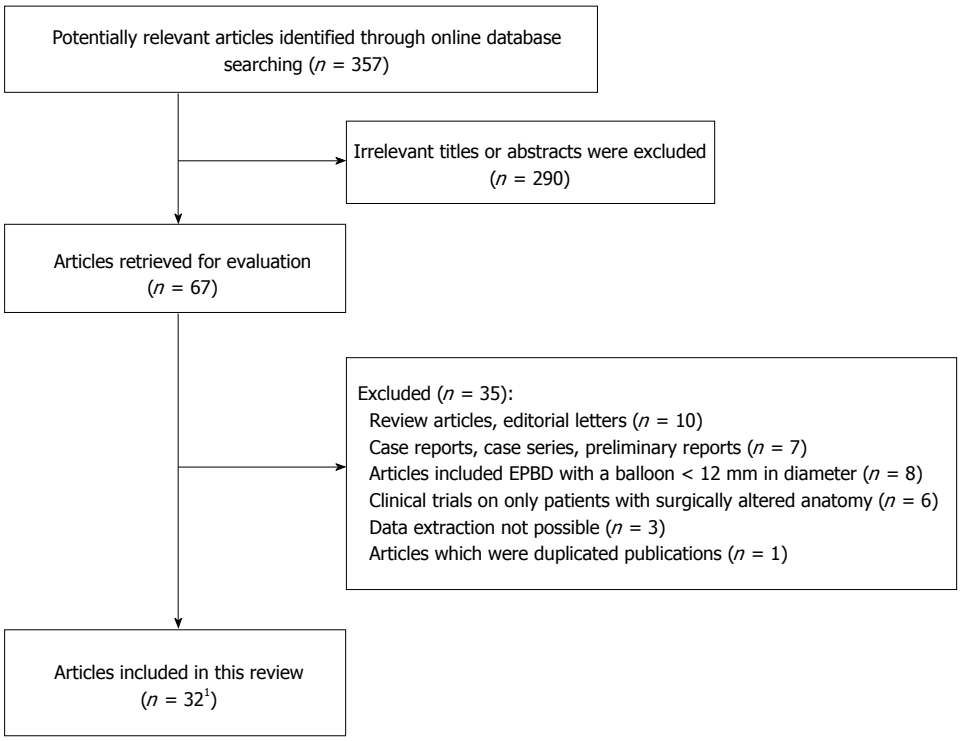

Figure 1 Flow-chart of literature search and evaluation.[1]Data from one article of a large scaled multi-center study was re-grouped into two; endoscopic papillary large balloon dilation with and without endoscopic sphincterotomy, and the outcomes were re-analyzed separately.

incision: (1) "large" if EST was completed to anywhere between two-thirds of the total length of the ampulla and up until the major horizontal fold crossing the intramural portion of the bile duct or if the extent of EST was described under such terms as "full incision EST"[40,46], "full-EST"[43], "maximum EST"[23], "major EST"[49], "complete EST"[29], "standard EST"[30,36] or "normal EST"[44]; and (2) "limited" if EST was made from the orifice of the ampulla proximally up to, but no exceeding two-thirds of the ampulla or if the extent of EST was described under such terms as "mid-incision EST"[22,28,38], "medium EST"[28], "middle EST"[48], "mid-EST"[43], "small EST"[24,42], "minor EST"[25] or "limited EST"[41,47,48].

Initial success was defined as complete bile duct stone clearance when only one session of EPLBD was performed whether EML as an adjunctive procedure was used or not. Overall success was defined as overall complete bile duct stone clearance by using EPLBD whether EML as an adjunctive procedure was used or not, with the exception of using other lithotripsies such as electrohydraulic lithotripsy and laser lithotripsy, irrespective of the number of EPLBD sessions. Success of EPLBD without EML was defined as complete stone clearance without the assistance of EML by using EPLBD irrespective of the number of EPLBD sessions. The rate of use of EML was defined as the rate for using EML as an adjunctive procedure to remove bile duct stones in all cases irrespective of the number of EPLBD sessions. Adverse events were classified and graded according to the consensus criteria proposed by Cotton *et al*[3].

STATISTICAL ANALYSIS

Statistical analyses were done using SPSS version 18.0 software (SPSS Inc., Chicago, Illinois, United States). The significance of difference for categorical variables was determined using either chi-square test or Fisher's exact test and a logistic regression analysis was performed for multiple comparisons in the statistically significant categorical variables that have more than two subgroups. Quantitative data were analyzed by either unpaired Student's *t* test or Mann-Whitney test, and presented as the mean ± SD. A *P* value below 0.05 was regarded as statistically significant.

EPLBD COMBINED WITH EST

A total of 2511 procedures in 2503 patients were included in this review from 30 published original articles, made up of 23 retrospective studies, 4 prospective studies and 3 prospective randomized controlled studies. The baseline clinical characteristics of the patients are described in Table 1. Periampullary diverticulum, which was provided in 25 studies, was noted in 36.7%. Prior EST, which was provided in 28 studies, was done in 20.2%. Patients with surgically altered anatomy of the upper gastrointestinal tract, such as Billroth I or Ⅱ surgery and Roux-en-Y anastomosis were included in 2.4% from 20 studies.

Patient outcomes

Based on the size of EST, EPLBD was performed in 10 studies mainly when stone removal had failed with the standard techniques after a large EST, in 13 studies after a limited EST mainly if it is speculated that the stone size is too large to be removed using the standard techniques, in 4 studies without additional EST if they had a previous history of EST, and in one multi-center study after variable sizes of EST. Twenty four studies described time duration of inflated balloon using a dilating balloon with a diameter of 12 to 20 mm which varied from 10 to 180 s, most of which were less than 60 s with the exception

Table 1 Baseline clinical characteristics of the patients undergoing endoscopic papillary large balloon dilation with endoscopic sphincterotomy *n* (%)

Ref.	Study design	No. of procedures, No. of patients	Mean age, year	No. of periampullary diverticulum	Mean size of largest stone, mm	Range of stone size, mm	Prior EST	Altered anatomy
Ersoz *et al*[21]	R	58	NA	4 (6.9)	NA	12-28	14	0
Hwang *et al*[22]	R	30	71.3	6 (20.0)	21.6	15-35	0	NA
Maydeo *et al*[23]	P	60	58.0	0 (0.0)	16.0	12-20	0	0
Minami *et al*[24]	R	88	74.0[1]	NA	14.0	> 12	0	NA
Heo *et al*[25]	RCT	100	64.4	49 (49.0)	16.0	NA	0	0
Lee *et al*[26]	R	55	70.8	16 (29.1)	20.8	15.4-35.5	0	B-Ⅱ:2
Kim *et al*[27]	R	35	66.9	9 (25.7)	26.1	12-50	14	NA
Lee *et al*[28]	R	41	72.2	21 (51.2)	18.2	10-45	0	B-Ⅱ:2, R-Y:2
Misra *et al*[29]	R	50	40.1	NA	NA	< 15-25	0	NA
Attasaranya *et al*[30]	R	107, 103	70.1	36 (35.0)	13.0[1]	10-30	50	B-Ⅱ:6
Espinel *et al*[31]	P	93	76.5	30 (32.2)	13.4	5-30	42	B-Ⅱ:4
Itoi *et al*[32]	R	53	75.3	25 (47.2)	14.8	10-28	0	0
Kim *et al*[33]	RCT	27	70.3	9 (33.3)	20.8	15-38.3	0	0
Itoi *et al*[34]	R	18	79.1	9 (50.0)	16.7	13-21	0	B-Ⅰ:1
Kurita *et al*[35]	R	24	82.0[1]	18 (75.0)	16.5[1]	12-33	24	NA
Ghazanfar *et al*[36]	P	84	48.4	NA	14.7	10-32	0	NA
Kim *et al*[37]	R	70	68.7	24 (34.3)	12.5	5-30	70	NA
Youn *et al*[38]	R	101	69.1	12 (11.9)	21.8	7-52	0	B-Ⅰ:2, B-II:3
Kim *et al*[39]	R	72	69.3	41 (56.9)	NA	> 10	0	0
Stefanidis *et al*[40]	RCT	45	69.4	NA	NA	12-20	0	0
Rebelo *et al*[41]	R	30	68.0	7 (23.3)	17.0[1]	12-30	4	NA
Sakai *et al*[42]	R	59	76.7	27 (45.8)	15.0	10-28	21	B-Ⅰ:3, B-Ⅱ:2
Park *et al*[43]	R	633	72.7	246 (39.1)	15.4	10-38.4	NA[2]	B-Ⅱ:20
Poincloux *et al*[44]	R	64, 62	77.0	15 (24.2)	NA	NA	0	NA
Hwang *et al*[45]	R	69	68.2	33 (47.8)	16.5	NA	0	0
Paspatis *et al*[46]	RCT	124	74.9	21 (16.9)	15.7	NA	NA[2]	0
Rosa *et al*[47]	R	68	70.8	NA	16.8	NA	0	0
Yang *et al*[48]	R	171, 169	69.3	73 (43.2)	15.0[1]	10-45	32	B-Ⅱ:1
Yoon *et al*[49]	P	52	68.1	19 (36.5)	20.1	12-40	52	0
Harada *et al*[50]	R	30	78.0	23 (76.7)	18.0	10-39	30	NA
Total		2511, 2503		773 (36.7)		5-45	353 (20.2)	48 (2.4)

[1]Median value; [2]Studies that included patients with a history of prior endoscopic sphincterotomy, but their exact numbers were not described. EST: Endoscopic sphincterotomy; R: Retrospective; P: Prospective; RCT: Randomized controlled trial; NA: Not available; B-Ⅰ: Billroth-Ⅰ anastomosis; B-Ⅱ: Billroth-Ⅱ anastomosis; R-Y: Roux-en-Y anastomosis.

of 3 studies[27,43,49] (Table 2). The initial success rate was 84.0% (range 61.9%-100%), which was provided in only 24 studies, thirteen of which studies were designed to include cases where EML was performed along with the first session of EPLBD. The mean number of EPLBD sessions for complete stone clearance was 1.2. The success rate of EPLBD without EML, the rate of use of EML, and the overall success rate, which were provided from all 30 studies, were 83.2% (59.6%-100%), 14.1% (0%-38.6%) and 96.5% (79.7%-100%), respectively (Table 2).

Adverse events

The overall rate of adverse events following EPLBD with EST was 8.3% (0%-17.0%), the majority of which were of mild to moderate severity. Adverse events were classified as pancreatitis, bleeding, perforation, and others (Table 3), and graded accordingly to severity as found in Table 4. Pancreatitis occurred in 2.4% (0%-13.2%), all cases of which were of mild to moderate severity (98.4%), except for one fatal case who had had a history of severe pancreatitis[46]. Bleeding occurred in 3.6% (0%-8.6%), but it mostly was of mild to moderate severity (94.5%). Four problematic bleedings, including 2 severe and 2 fatal cases, were reported in 4 studies[29,30,36,43]; two were successfully managed with angiography and surgery, respectively, and the other two had expired due to post-EPLBD massive bleeding. Perforation occurred in 0.6% (0%-2.8%). Six problematic perforations (5 duodenum and 1 cystic duct), including 3 severe and 3 fatal cases, were reported in 3 studies[30,43,45]; two with duodenal perforation were successfully managed with surgery and one with cystic duct perforation with percutaneous drainage, and the other three expired due to septic shock and multi-organ failure (2) and cardiogenic shock (1). Other adverse events were noted in 1.7% (0%-14.8%), including cholangitis (14), hypotension (10), pain (4), intramural dissection (3), pneumonia (3), basket impaction (2), sepsis (2), cholecystitis (1), injured bile duct (1), and hypoxia (1). All of these cases were successfully managed with conservative treatment, except for all basket impaction cases who received surgery.

EPLBD WITHOUT EST

A total of 413 patients who each received EPLBD without EST were included in this review from 3 published

Table 2 Procedure characteristics and outcomes of endoscopic papillary large balloon dilation with endoscopic sphincterotomy *n* (%)

Ref.	Size of EST	Balloon size, mm	Duration of inflated balloon, s	Initial success	No. of sessions, mean	Success without EML	Use of EML	Overall success
Ersoz *et al*[21]	Large	12-20	20-45	48 (82.8)	1.17	54 (93.1)	4 (6.9)	58 (100)
Hwang *et al*[22]	Limited	15-18	30-60	NA	NA	30 (100.0)	0 (0.0)	30 (100)
Maydeo *et al*[23]	Large	12-20	30	57 (95.0)	1.05	57 (95.0)	3 (5.0)	60 (100)
Minami *et al*[24]	Limited	20	NA	87 (98.9)	1.00	87 (98.9)	1 (1.1)	88 (100)
Heo *et al*[25]	Limited	12-20	60	83 (83.0)	1.12	90 (90.0)	8 (8.0)	97 (97.0)
Lee *et al*[26]	Limited	15-20	30-60	NA	NA	52 (94.5)	3 (5.5)	55 (100)
Kim *et al*[27]	Limited	12-20	60-90	NA	NA	22 (63.1)	9 (25.7)	31 (88.6)
Lee *et al*[28]	Limited	13-20	20-60	35 (85.3)	1.20	37 (90.3)	4 (9.8)	41 (100)
Misra *et al*[29]	Large	15-20	30-45	NA	NA	45 (90.0)	5 (10.0)	50 (100)
Attasaranya *et al*[30]	Large	12-18	NA	102 (95.3)[1]	1.00	78 (72.9)	29 (27.1)	102 (95.3)
Espinel *et al*[31]	Large	12-20	30-45	93 (100.0)[1]	1.00	90 (96.8)	3 (3.2)	93 (100)
Itoi *et al*[32]	Large	15-20	15-30	51 (96.2)[1]	1.04	50 (94.3)	3 (5.7)	53 (100)
Kim *et al*[33]	Limited	15-18	NA	23 (85.2)[1]	1.27	18 (66.7)	9 (33.3)	27 (100)
Itoi *et al*[34]	Large	15-18	10	17 (94.4)	1.06	14 (77.8)	4 (22.2)	18 (100)
Kurita *et al*[35]	Prior	15-20	30	23 (95.8)	1.00	23 (95.8)	1 (4.2)	23 (95.8)
Ghazanfar *et al*[36]	Large	15-18	NA	52 (61.9)	1.28	67 (79.7)	0 (0.0)	67 (79.7)
Kim *et al*[37]	Prior	12-18	20-60	68 (97.1)	1.02	69 (98.6)	1 (1.4)	70 (100)
Youn *et al*[38]	Limited	15-20	30-60	93 (92.1)[1]	1.08	94 (93.1)	7 (6.9)	101 (100)
Kim *et al*[39]	Limited	12-20	30	63 (87.5)[1]	1.14	64 (88.9)	6 (8.3)	70 (97.2)
Stefanidis *et al*[40]	Large	15-20	10-12	44 (97.7)	1.00	44 (97.7)	0 (0.0)	44 (97.7)
Rebelo *et al*[41]	Limited	12-18	60	25 (83.3)[1]	1.14	23 (76.7)	6 (20.0)	29 (96.7)
Sakai *et al*[42]	Limited	12-20	NA	49 (83.1)[1]	1.30	51 (86.4)	8 (13.6)	57 (96.6)
Park *et al*[43]	Variable	12-20	30-180	357[3] (65.4)[1]	1.46	484[4] (78.4)	123[4] (19.9)	602[4] (97.6)
Poincloux *et al*[44]	Large	15-20	30-60	62 (96.9)	1.05	61 (95.3)	3 (4.7)	64 (100)
Hwang *et al*[45]	Limited	12-20	60	65 (94.2)[1]	1.02	51 (73.9)	18 (26.1)	66 (95.7)
Paspatis *et al*[46]	Large	15-20	30-60	NA	NA	102 (81.8)	4 (3.2)	106 (85.0)
Rosa *et al*[47]	Limited	12-18	60	56 (82.4)[1]	1.10	55 (80.9)	10 (14.7)	65 (95.6)
Yang *et al*[48]	Limited	12-18	NA	163 (95.3)[1]	1.00	102 (59.6)	66 (38.6)	163 (95.3)
Yoon *et al*[49]	Prior	12-20	60-120	NA	1.70	36 (69.2)	12 (23.1)	48 (92.4)
Harada *et al*[50]	Prior	15-20	30	29 (96.7)[1]	1.00	27 (90.0)	3 (10.0)	29 (96.7)
Total		12-20	10-180	1745 (84.0)	1.20[2]	2077 (83.2)	353 (14.1)	2407 (96.5)

[1]Studies which were designed to include cases where endoscopic mechanical lithotripsy was performed along with the first session of endoscopic papillary large balloon dilation; [2]Calculated by dividing total number of procedures into total number of sessions which was calculated by multiplying each mean number of session with each number of procedures; [3]Total number of procedures was 546 due to missing data; [4]Total number of procedures was 617 due to missing data. EST: Endoscopic sphincterotomy; EML: Endoscopic mechanical lithotripsy; Prior: Prior endoscopic sphincterotomy; NA: Not available.

original articles, all of which were retrospective studies. The baseline clinical characteristics of the patients are described in Table 5. Mean age was 71.8, periampullary diverticulum was noted in 33.2% of the patients, the mean size of the largest stone was 15.4 mm, the range of stone size was 10 mm up to 37 mm, and patients with Billroth Ⅱ surgery were included in 2.7%.

Patient outcomes

EPLBD without EST was performed using a dilating balloon with a diameter of 12 to 20 mm in all 3 studies with time duration of inflated balloon of 30 s up to 180 s. The initial success rate was 76.2% (74.1%-91.9%), but two of the 3 studies were designed to include cases where EML was performed along with the first session of EPLBD. The mean number of EPLBD sessions for complete stone clearance was 1.27. The success rate of EPLBD without EML, the rate of use of EML, and the overall success rate were 76.7% (76.0%-80.6%), 21.6% (19.4%-21.7%), and 97.2% (96.8%-97.4%), respectively (Table 6).

Adverse events

The overall rate of adverse events following EPLBD without EST was 7.0% (2.6%-7.7%), the majority of which were of mild to moderate severity. Adverse events were classified as pancreatitis, bleeding, perforation, and others (Table 7), and graded accordingly to severity as found in Table 4. No cases of severe or fatal adverse events were reported. Pancreatitis and bleeding occurred in 3.9% (2.6%-6.4%) and 1.9% (0%-2.6%), respectively, all cases of which were of mild to moderate severity. Perforation occurred in two cases, 0.5% (0%-0.6%), both of which were of moderate severity, which were successfully managed with conservative management. As other adverse events, only 3 cases of mild cholangitis were reported from one multicenter study[43].

COMPARISON BETWEEN EPLBD WITH EST AND EPLBD WITHOUT EST

Comparison between patients who received EPLBD with

Table 3 Adverse events of endoscopic papillary large balloon dilation with endoscopic sphincterotomy *n* (%)

Ref.	Overall AEs	Pancreatitis	Bleeding	Perforation	Others	AE-related surgery	AE-related death
Ersoz *et al*[21]	9 (15.5)	2 (3.4)	5 (8.6)	0 (0.0)	2 (3.4)	0 (0.0)	0 (0.0)
Hwang *et al*[22]	1 (3.3)	0 (0.0)	1 (3.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Maydeo *et al*[23]	5 (8.3)	0 (0.0)	5 (8.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Minami *et al*[24]	15 (17.0)	1 (1.1)	1 (1.1)	0 (0.0)	13 (14.8)	0 (0.0)	0 (0.0)
Heo *et al*[25]	5 (5.0)	4 (4.0)	0 (0.0)	0 (0.0)	1 (1.0)	0 (0.0)	0 (0.0)
Lee *et al*[26]	2 (3.6)	0 (0.0)	2 (3.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Kim *et al*[27]	1 (2.8)	0 (0.0)	0 (0.0)	1 (2.8)	0 (0.0)	0 (0.0)	0 (0.0)
Lee *et al*[28]	3 (7.2)	2 (4.8)	1 (2.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Misra *et al*[29]	7 (14.0)	4 (8.0)	3 (6.0)	0 (0.0)	0 (0.0)	1 (2.0)	0 (0.0)
Attasaranya *et al*[30]	6 (5.6)	0 (0.0)	2 (1.9)	1 (0.9)	3 (2.8)	1 (0.9)	0 (0.0)
Espinel *et al*[31]	2 (2.2)	1 (1.1)	1 (1.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Itoi *et al*[32]	2 (3.8)	1 (1.9)	0 (0.0)	0 (0.0)	1 (1.9)	0 (0.0)	0 (0.0)
Kim *et al*[33]	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Itoi *et al*[34]	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Kurita *et al*[35]	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Ghazanfar *et al*[36]	6 (7.1)	3 (3.6)	3 (3.6)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.2)
Kim *et al*[37]	1 (2.3)	1 (2.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Youn *et al*[38]	7 (6.9)	2 (2.0)	2 (2.0)	1 (1.0)	2 (2.0)	0 (1.0)	0 (1.0)
Kim *et al*[39]	6 (8.3)	5 (6.9)	0 (0.0)	0 (0.0)	1 (1.4)	0 (0.0)	0 (0.0)
Stefanidis *et al*[40]	2 (4.4)	1 (2.2)	1 (2.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Rebelo *et al*[41]	4 (13.3)	1 (3.3)	0 (0.0)	0 (0.0)	3 (10.0)	0 (0.0)	0 (0.0)
Sakai *et al*[42]	4 (6.8)	0 (0.0)	1 (1.7)	1 (1.7)	2 (3.4)	0 (0.0)	0 (0.0)
Park *et al*[43]	71 (11.2)	13 (2.1)	48 (7.6)	7 (1.1)	3 (0.4)	2 (0.3)	4 (0.6)
Poincloux *et al*[44]	9 (14.1)	2 (3.1)	5 (7.8)	0 (0.0)	2 (3.1)	0 (0.0)	0 (0.0)
Hwang *et al*[45]	5 (7.2)	3 (4.3)	0 (0.0)	1 (1.4)	1 (1.4)	2 (2.9)	0 (0.0)
Paspatis *et al*[46]	17 (13.7)	4 (3.2)	6 (4.8)	2 (1.6)	5 (4.1)	0 (0.0)	1 (0.8)
Rosa *et al*[47]	10 (14.7)	9 (13.2)	0 (0.0)	0 (0.0)	1 (1.5)	0 (0.0)	0 (0.0)
Yang *et al*[48]	8 (4.7)	2 (1.2)	4 (2.4)	1 (0.6)	1 (0.6)	0 (0.0)	0 (0.0)
Yoon *et al*[49]	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Harada *et al*[50]	1 (3.3)	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.3)	0 (0.0)	0 (0.0)
Total	809 (8.3)	61 (2.4)	91 (3.6)	15 (0.6)	42 (1.7)	6 (0.2)	6 (0.2)

AE: Adverse event.

Table 4 Comparison between endoscopic papillary large balloon dilation with and without endoscopic sphincterotomy *n* (%)

	EPLBD with EST	No. of studies	EPLBD without EST	No. of studies	*P* value
No. of procedures	2511	30	413	3	
Mean of mean age, yr	69.6 ± 8.6[1]	29	70.3 ± 2.3[1]	3	0.808
Periampullary diverticulum	773 (36.7)	23	122 (33.2)	2	0.186
Initial success	1745 (84.0)	24	285 (76.2)	3	< 0.001
Success without EML	2077 (83.2)	30	306 (76.7)	3	0.001
Use of EML	353 (14.1)	30	86 (21.6)	3	< 0.001
Overall success	2407 (96.5)	30	388 (97.2)	3	0.432
Overall adverse events	209 (8.3)	30	29 (7.0)	3	0.370
Pancreatitis, total; M/Mod/S/F	61; 51/9/0/1 (2.4)	30	16; 14/2/0/0 (3.9)	3	0.089
Bleeding, total; M/Mod/S/F	91; 75/11/2/2 (3.6)[2]	30	8; 7/1/0/0 (1.9)	3	0.079
Perforation, total; M/Mod/S/F	15; 3/6/3/3 (0.6)	30	2; 0/2/0/0 (0.5)	3	1.000
Other adverse events	42 (1.7)	30	3; 3/0/0/0 (0.7)	3	0.148
AE-related surgery	6 (0.2)	30	0 (0)	3	1.000
AE-related death	6 (0.2)	30	0 (0)	3	1.000

[1]mean ± SD; [2]One case of bleeding was not graded for severity. M: Mild; Mod: Moderate; S: Severe; F: Fatal; EPLBD: Endoscopic papillary large balloon dilation; EST: Endoscopic sphincterotomy; EML: Endoscopic mechanical lithotripsy; AE: Adverse event.

EST and those who received EPLBD without EST were summarized in Table 4. Mean age and the rate of periampullary diverticulum showed no significant difference between both procedures. Mean number of EPLBD session and the overall success rate were not significantly different between both procedures, but the initial success rate (84.0% *vs* 76.2%, $P < 0.001$) and the success rate of EPLBD without EML (83.2% *vs* 76.7%, $P = 0.001$) were significantly higher in patients who received EPLBD with EST than in those who received EPLBD without EST, while the rate of use of EML (14.1% *vs* 21.6%, $P < 0.001$) were significantly lower in patients who received EPLBD with EST. Overall adverse events, pancreatitis, bleeding, perforation, other adverse events, the rate of surgery for

Table 5 Baseline clinical characteristics of the patients on endoscopic papillary large balloon dilation without endoscopic sphincterotomy *n* (%)

Ref.	Study design	No. of procedures	Mean age, yr	No. of periampullary diverticulum	Mean size of largest stone, mm	Range of stone size, mm	Altered anatomy
Jeong *et al*[51]	R	38	68	NA	17.7	12-31	0
Hwang *et al*[45]	R	62	70.4	16 (25.8)	15.7	12-26	0
Park *et al*[43]	R	313	72.6	106 (34.6)	15.0	10-37	B-Ⅱ:11
Total		413	71.8[1]	122 (33.2)	15.4[2]	10-37	11 (2.7)

[1]Calculated by dividing total number of procedures into total number of the parameter which was calculated by multiplying each mean value with each number of procedures; [2]A retrospective multicenter study where missing data are present in each analyzed variable. R: Retrospective; B-Ⅱ: Billroth-Ⅱ anastomosis.

Table 6 Procedure characteristics and outcomes of endoscopic papillary large balloon dilation without endoscopic sphincterotomy *n* (%)

Ref.	Balloon size, mm	Duration of inflated balloon, s	Initial success	No. of sessions, mean	Success without EML	Use of EML	Overall success
Jeong *et al*[51]	15-18	60	25 (65.8)	1.20	29 (76.3)	9 (23.7)	37 (97.4)
Hwang *et al*[45]	12-20	60	57 (91.9)[1]	1.05	50 (80.6)	12 (19.4)	60 (96.8)
Park *et al*[43]	12-20	30-180	203[3] (74.1)[1]	1.33	227[4] (76.0)	65[4] (21.7)	291[4] (97.3)
Total	12-20	30-180	285 (76.2)	1.27[2]	306 (76.7)	86 (21.6)	388 (97.2)

[1]Studies which were designed to include cases where endoscopic mechanical lithotripsy was performed along with the first session of endoscopic papillary large balloon dilation; [2]Calculated by dividing total number of procedures into total number of sessions which was calculated by multiplying each mean number of session with each number of procedures; [3]Total number of procedures was 274 due to missing data; [4]Total number of procedures was 299 due to missing data. EML: Endoscopic mechanical lithotripsy.

Table 7 Adverse events of endoscopic papillary large balloon dilation without endoscopic sphincterotomy *n* (%)

Ref.	Overall AEs	Pancreatitis	Bleeding	Perforation	Others	AE-related surgery	AE-related death
Jeong *et al*[51]	1 (2.6)	1 (2.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Hwang *et al*[45]	4 (6.4)	4 (6.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Park *et al*[43]	24 (7.7)	11 (3.5)	8 (2.6)	2 (0.6)	3 (1.0)	0 (0.0)	0 (0.0)
Total	29 (7.0)	16 (3.9)	8 (1.9)	2 (0.5)	3 (0.7)	0 (0.0)	0 (0.0)

AE: Adverse event.

Table 8 Comparison of adverse events among endoscopic papillary large balloon dilation with large, limited and without endoscopic sphincterotomy *n* (%)

	EPLBD with large EST	EPLBD with limited EST	EPLBD without EST	*P* value
No. of procedures	756	946	413	
Overall adverse event	65 (8.6)	71 (7.5)	29 (7.0)	0.568
Pancreatitis	18 (2.4)	29 (3.1)	16 (3.9)	0.349
Bleeding	31 (4.1)	12 (1.3)	8 (1.9)	0.001[1]
Perforation	3 (0.4)	5 (0.5)	2 (0.5)	1.000
Other adverse events	13 (1.7)	25 (2.6)	3 (0.7)	0.054
AE-related surgery	2 (0.3)	2 (0.2)	0 (0.0)	0.832
AE-related death	2 (0.3)	0 (0.0)	0 (0.0)	0.166

[1]EPLBD with large EST *vs* EPLBD with limited EST, $P < 0.001$; EPLBD with large EST *vs* EPLBD without EST, $P = 0.049$; EPLBD with limited EST *vs* EPLBD without EST, $P = 0.35$. EPLBD: Endoscopic papillary large balloon dilation; EST: Endoscopic sphincterotomy; AE: Adverse event.

adverse events, and fatal adverse events were not significantly different between both procedures.

We compared the rates of adverse events among 3 kinds of EPLBD procedures which we classified based on the extent of ampullary incision of the EST; large EST, limited EST and no EST (Table 8). There were no significant differences among the 3 EPLBD procedures in the rates of the overall adverse events, pancreatitis, perforation, other adverse events, and adverse events related to surgery and death, but the rate of bleeding was significantly higher in EPLBD with large EST, compared with EPLBD with limited EST ($P < 0.001$, OR = 3.33) or without EST ($P = 0.049$, OR = 2.17), but no significant difference between EPLBD with limited EST and

Table 9 Comparison among endoscopic sphincterotomy, endoscopic papillary balloon dilation, and endoscopic papillary balloon dilation with endoscopic sphincterotomy *n* (%)

	EST[1]	EPBD[1]	No. of studies	EPLBD with EST	No. of studies	*P* value
No. of procedures	890	878	15	2511	30	
Mean age, range, yr	47-71	49-75	15	40-82	29	
Mean stone size, range, mm	7.3-16.9	7-15.6	15	5-45	25	
Initial success	322 (80.9)	285 (73.5)	7	1745 (84.0)	24	< 0.001
Use of EML	121 (13.3)	162 (19.6)	13	353 (14.1)	30	< 0.001
Overall success	776 (95.3)	733 (90.1)	13	2407 (96.5)	30	< 0.001
Overall adverse events	113 (12.7)	106 (12.1)	15	209(8.3)	30	< 0.001
Pancreatitis	36 (4.3)	71 (8.6)	14	61 (2.4)	30	< 0.001
Bleeding	33 (4.8)	1 (0.1)	12	91 (3.6)	30	< 0.001
Perforation	3 (0.5)	2 (0.3)	9	15 (0.6)	30	0.941
AE-related death	2 (0.3)	4 (0.7)	7	6 (0.24)	30	0.152

[1]Results of a meta-analysis by Weinberg *et al*[18]. EST: Endoscopic sphincterotomy; EPBD: Endoscopic papillary balloon dilation; EPLBD: Endoscopic papillary large balloon dilation; EML: Endoscopic mechanical lithotripsy.

without EST ($P = 0.35$).

DISCUSSION

Standard basket and balloon techniques after EST are most commonly used for the removal of bile duct stones with overall success rates of more than 80% to 90%[88-92]. When it fails due to the stone size being larger than the widened ampullary orifice by performing EST or the distal common bile duct, additional endoscopic procedures, mainly EML, are usually required for complete stone clearance[93-97]. However, EML proved to be a time-consuming and challenging technique[11,98,99]. EPLBD has been widely used as the alternative to EST with EML for the removal of large or difficult bile duct stones. EPLBD was initially performed when the standard techniques failed after a large EST[21,23], but recently it has been performed after a limited EST or sometimes without EST, even before attempting trials the standard technique with a large EST. Such procedure is usually performed when it is speculated that the size of the stone is too large for it to be removed using the standard techniques after a large EST and on the assumption that it would reduce the incidence rate of potential serious adverse events of a large EST such as bleeding and bile duct perforation.

The initial success rate and the overall success rate were 84.0% and 96.5%, respectively, in EPLBD with EST in this review, while the results showed 80.9% and 95.3% in EST alone and 73.5% and 90.1% in EPBD alone, respectively, in a previous meta-analysis[18] (Table 9). When we compared these results, the initial success rate was significantly lower in EPBD alone than EPLBD with EST ($P < 0.001$, OR = 1.89) and EST alone ($P = 0.013$, OR = 1.53), but showing no significant differences between EPLBD with EST and EST alone ($P = 0.131$); the overall success rate was also significantly lower in EPBD alone than EPLBD with EST ($P < 0.001$, OR = 2.72) and EST alone ($P = 0.001$, OR = 2.03), and showing also no significant differences between EPLBD with EST and EST alone ($P = 0.141$). However, a comparison between these meta-analysis results and ours is somewhat contradictory because their meta-analysis was of relatively small bile duct stones. Furthermore, the initial success rate in this review was statistically flawed, because studies included were designed heterogeneously based on different definitions, some of which included cases where EML was performed along with the first session of EPLBD. There were only 4 comparison studies, including 2 prospective randomized studies[25,33] and 2 retrospective studies[32,39], done for the evaluation of outcomes between EPLBD with EST and EST alone with the assistance of EML in patients with large or difficult bile duct stones. However, these studies failed to show any differences in the initial success rate and the overall success rate between both procedures, except one retrospective study[32], where EPLBD with EST was superior to EST alone only in the initial success rate, not the overall success rate[32]. The initial success rate in EPLBD without EST in this review was significantly lower, compared with that in EPLBD with EST, most likely due to the opening of the orifice retracting almost immediately back to its original size which is commonly seen in EPBD alone. However, the overall success rate showed no significant difference between both of them.

The intended purpose of EPLBD was to simplify removing large or difficult bile duct stones without additional adverse events to EST alone or EPBD alone, and contemplated major advantages were that it would reduce both the need of EML and the procedure time, increasing the success rates of stone removal, compared with EST alone and EPBD alone. This is believed to be because the wider ampullary orifice, made when using EPLBD, would facilitate in the easier extraction of relatively large bile duct stones. In addition, it may also reduce potential EML-related adverse events, such as basket impaction and bile duct injury. However, the frequency of EML use in EPLBD might be related to various factors, such as the diameter of dilating balloon used, discrepancy in the size between the stone and the ampullary orifice or the distal bile duct, and the shape of the stone and the bile duct. The rate of use of EML was 14.1% in EPLBD with EST with a wide range of 0% to 38.6% in this review. It

showed similar results in EST alone of 13.3% in a previous meta-analysis[18], but significantly lower than EPLBD without EST of 21.6% from this review and EPBD alone of 19.6% in a previous meta-analysis[18]. In 4 comparison studies between EPLBD with EST and EST alone, there were conflicting results concerning the use of EML for the removal of large or difficult bile duct stones; two prospective randomized studies reported no significant difference in the use of EML[25,33], on the contrary to two retrospective studies[32,39]. These studies overlooked one important fact that the rate of use of EML when tallied against the number of patients requiring EML, could not help but be similar between both procedures, because EML was still needed in patients where the size of the stones exceeded the size of the widened ampullary orifice even after EPLBD. However, the need for repeated EML would be reduced due to a wider ampullary orifice, if the stones were fragmented mostly by one session of EML following EPLBD. Thus, for a more accurate evaluation about the rate of use of EML, it should be calculated based on the frequency of EML use in each patient who underwent EPLBD, not the number of patients requiring EML. Mean procedure time was evaluated in two of these 4 comparison studies; one prospective randomized study failed to show any difference between EPLBD with EST and EST alone[33], while the other retrospective study showed a shorter procedure time in EPLBD with EST[32]. Large-scale, prospective multicenter comparison studies will be needed to confirm advantages of EPLBD in the frequency of EML use and procedure time.

In results of adverse events following EPLBD in this review, adverse events in EPLBD without EST showed no significant difference compared with those in EPLBD with EST. The most common adverse event in each procedure was bleeding with a mean rate of 3.6% in EPLBD with EST and pancreatitis with a mean rate of 3.9% in EPLBD without EST. Our results showed definite evidence that EPLBD with and even without EST, did not increase the risk of serious pancreatitis, as more frequently seen in EPBD using small-diameter balloons ($\leqslant$ 10 mm)[18-20]. It is no doubt that the mechanism of pancreatitis would be different in EPLBD, compared with EPBD, although its mechanism still remains unclear, a major etiologic factor of pancreatitis. The most serious adverse event was bile duct perforation in EPLBD with EST. The following shows the comparison of adverse events between results of a previous meta-analysis[18] in EST alone and EPBD alone and those of our review in EPLBD with EST (Table 9); the rate of overall adverse events was significantly lower in EPLBD with EST than EST alone ($P < 0.001$, OR = 1.60) and EPBD alone ($P = 0.001$, OR = 1.51); the rate of pancreatitis was significantly lower in EPLBD with EST than EST alone ($P = 0.006$, OR = 1.80) and EPBD alone ($P < 0.001$, OR = 3.77); the rate of bleeding was not significantly different between the EPLBD with EST and EST alone ($P = 0.164$) and was significantly lower in EPBD alone than EPLBD with EST ($P = 0.001$, OR = 25.27) and EST alone ($P = 0.001$, OR = 33.75); the rate of perforation and the rate of adverse event-related death showed no significant differences among the 3 procedures ($P = 0.941$ and $P = 0.152$, respectively). However, within 4 comparison studies on adverse events between EPLBD with EST and EST alone, each study showed no significant differences between both of them [25,32,33,39].

Major risk factors which are related to adverse events include procedure-related factors such as size of balloon, size of EST, and time duration of inflated balloon, and patient-related factors such as the existence of bile duct strictures, periampullary diverticulum, surgically altered anatomy, and a bleeding tendency. Park *et al*[43] reported that larger stone size more than 16mm in diameter, underlying cirrhosis, and full-length EST were independently associated with an increase in adverse events. The size of the balloon is the most important major factor in ensuring a success of EPLBD and a reduction of adverse events[53]. As the ampullary orifice becomes wider as a result of balloon dilation, stone removal becomes easier. However, choosing an inappropriately oversized balloon increases the risk of adverse events, such as perforation or bleeding due to blood vessel injury[53]. Interestingly, a multicenter study by Park *et al*[43] reported that balloons larger than 14 mm in diameter were independently associated with a decreased risk of pancreatitis, projecting that only simple stretching of the ampullary orifice or direct blockage of the pancreatic orifice by compression of large-diameter balloons is not a major etiologic factor of pancreatitis following EPLBD.

The intended maximal target diameter of a dilating balloon for EPLBD should be determined based on the size of the stone and the size of the distal bile duct proximal to the tapered segment[26,55,100], but must never exceed the diameter of the distal bile duct to prevent bile duct perforation[43,53]. A 12- to 20-mm diameter balloon for pyloric use (CRE^{TM} wire-guided balloon dilator, Boston Scientific, Natick, Massachusetts, United States) is mostly used to dilate the duodenal ampulla during EPLBD, each of which gradually inflates in 3 different diameter steps by increasing balloon inflation pressure. The balloon used should be selected with the 2nd or the 3rd diameter step being the intended maximal target diameter, and be inflated gradually, starting from a smaller diameter step of the balloon than the intended maximal target diameter. The balloon is slowly dilated until it reaches its 1st diameter step with gradual increment of balloon pressure to prevent sudden tearing of the ampullary roof. If the balloon is dilated without any difficulty with the disappearance of its central waist, it is then dilated gradually to its 2nd diameter step and then further up to its 3rd diameter step till its diameter reaches the intended maximal target diameter. If the central waist of the balloon does not disappear against the marked resistance of the bile duct or the patient indicates severe pain during balloon inflation at any step, further balloon inflation must be ceased for the prevention of bile duct perforation[43]. Lee *et al*[55] recommended based on personal experience that balloon

inflation should be discontinued if the balloon waist does not disappear even once it reaches 75% of the recommended maximal inflation pressure. In patients who are known to have obvious distal bile duct strictures, EPBLD should be avoided to prevent bile duct perforation[43,55]. If there is a suspicion of strictures, based on personal experience, we recommend pulling back a large retrieval balloon, that should be inflated up to approximately the same size as the distal bile duct, just up until the inside of the ampullary orifice. If there is no existence of a stricture, the suspected site of stricture should easily expand allowing the balloon to pass through without any resistance.

The extent of ampullary incision is another important major factor to prevent adverse events, such as bleeding and perforation. Theoretically, EPLBD with limited EST would have combined advantages to minimize major adverse events of both EST alone and EPBD alone, such as bleeding and perforation mainly in a large EST and pancreatitis mainly in EPBD[54]. In comparison of the 3 different EPLBD procedures based on the extent of ampullary incision of the preceding EST, which were classified into large, limited and no EST, it showed no significant differences among them in the rates of overall adverse events, pancreatitis, perforation and other adverse events. However, the rate of bleeding was significantly higher in EPLBD with large EST than in EPLBD with limited EST or without EST, but there was no significant difference between EPLBD with limited EST and without EST. Delayed fatal bleeding was noted in 2 patients who underwent a full-incision EST before EPLBD in this review. Delayed serious bleeding may occur if a large blood vessel located at the proximal part of the ampullary roof is severed during full-incision EST, not injury caused by stretching of the ampullary orifice using a large-diameter balloon. Therefore, EPLBD with large, especially full-incision EST should be avoided to prevent serious bleeding. In patients with prior EST, it is known that extended incision of the previous EST site can increase the risk of adverse events such as bleeding or perforation[3,6,8]. Therefore, almost all patients with prior EST did not receive repeated EST in this review. There were 3 retrospective studies and one prospective study about clinical trials of EPLBD using 12- to 20-mm large balloons on only patients with prior EST but without repeated EST, showing similar results in stone clearance and adverse events, compared with their counterpart studies in which all patients underwent no prior EST[35,37,49,50].

The main purpose of EST during EPLBD is not to make an incision of the duodenal ampulla long, but to control the direction of tearing during balloon dilation. A probable mechanism of a reduced pancreatitis rate in EPLBD with EST is believed to be that the radial force exerted by the dilating balloon shifts along the cutting direction made during EST toward the bile duct away from the pancreatic orifice, resulting in less periampullary injury around the pancreatic duct with a decreased risk of pancreatitis[21,24,43,101]. However, EST may be a limited role in preventing pancreatitis in EPLBD, because there was no evidence that EPLBD without EST increased the risk of pancreatitis in this review. So to explain this, we suggest the following hypothesis surrounding the mechanism of pancreatitis after EPLBD; manipulation of Dormia basket and retrieval balloon catheter as well as the frequency of EML in EPLBD with, or even without, EST, may be reduced due to a sufficiently widened ampullary orifice, resulting in less periampullary trauma or edema that occurs during stone extraction and eventually leading to a low risk of pancreatitis. On the contrary, its frequency in EPBD using small-diameter balloons is increased due to the ampullary orifice not widening enough, increasing the risk of pancreatitis[100].

Time duration of inflated balloon of the duodenal ampulla during EPLBD is mostly around 1 min in this review, after the intended maximal target diameter of the balloon was reached. One prospective randomized study revealed that 30 s of duration of inflated balloon was not different in adverse events, including pancreatitis, bleeding and perforation, to 60 s in EPLBD with EST[46]. The longer the time duration of inflated balloon did not seem to be related to an increase of the risk of adverse events, and the shorter the time duration of inflated balloon seems to be related to an increase of the risk of serious bleeding, due to insufficient compression by the balloon. Further studies are warranted to determine the optimal time duration of inflated balloon during EPLBD.

The patient-related factors related to adverse events include periampullary diverticulum, surgically altered anatomy, and a bleeding tendency. Patients with periampullary diverticulum were suitable for EPLBD. A retrospective comparison study in patients between with and without periampullary diverticulum, showed similar stone clearance rates and adverse events in both, following EPLBD with limited EST[76], and several studies reported that the presence of a periampullary diverticulum was not associated with a significant increased rate of adverse events such as pancreatitis, bleeding, or perforation[43,46,48,86]. There were 6 studies about clinical trials of EPLBD on only patients with surgically altered anatomy, such as Billroth Ⅱ surgery (5)[77-81] and Roux-en-Y anastomosis (1)[82], resulting in complete stone clearance in all patients with a low incidence of pancreatitis and bleeding. In patients with coagulopathy, EPLBD without EST may be useful, but should be undertaken cautiously[43,100], even though further studies are warranted. Park *et al*[43] reported that the size of the bile duct stone (≥ 16 mm) and presence of cirrhosis might be independent factors of bleeding. If serious bleeding from the ampulla occurs after balloon deflation, compression of the ampulla with re-ballooning can be done for several minutes till the bleeding stops.

Our recommendations for a successful EPLBD are as follows, based on the results in this review and personal experiences: (1) EPLBD with large, especially full-incision EST should be avoided to prevent serious bleeding; (2) EPLBD with limited EST is recommended to be

Table 10 Recommendations for successful endoscopic papillary large balloon dilation

1 EPLBD with large, especially full-incision EST should be avoided
2 EPLBD with limited EST is recommended to be performed, even before attempting trials of a standard technique with large EST, when the stone is seen to be too large on cholangiogram
3 EPLBD without EST may be useful in some patients with coagulopathy, periampullary diverticulum, or surgically altered anatomy
4 In patients with obvious distal bile duct strictures, EPBLD should be avoided. If there is a suspicion of strictures, using the pulling method of a large inflated retrieval balloon through the site is recommended to confirm an existence
5 The intended maximal target diameter of the balloon should be determined based on the diameter of the largest stones, but should not exceed the diameter of the distal bile duct
6 The balloon should always be inflated gradually, starting from a smaller diameter step of the balloon than the intended maximal target diameter
7 Further balloon inflation must be ceased, if the central waist of the balloon does not disappear or the patient indicates severe pain during balloon inflation at any step

EST: Endoscopic sphincterotomy; EPLBD: Endoscopic papillary large balloon dilation.

performed to reduce the risk of bleeding and perforation even before attempting trials of a standard technique with large EST, when the stone is seen to be too large on cholangiogram; (3) EPLBD without EST may be useful in some patients with coagulopathy, periampullary diverticulum, or surgically altered anatomy to reduce the risk of serious bleeding and perforation; (4) In patients with obvious distal bile duct strictures, EPBLD should be avoided to prevent perforation. If there is a suspicion of strictures, using the pulling method of a large inflated retrieval balloon through the site is recommended to confirm an existence; (5) The intended maximal target diameter of the balloon should be determined based on the diameter of the largest stones, but should not exceed the diameter of the distal bile duct to reduce the risk of perforation; (6) The balloon should always be inflated gradually, starting from a smaller diameter step of the balloon than the intended maximal target diameter; and (7) Further balloon inflation must be ceased to prevent perforation, if the central waist of the balloon does not disappear or the patient indicates severe pain during balloon inflation at any step (Table 10).

There are several limitations to this review. It was very difficult to analyze systematically the outcomes of EPLBD, because the results from each relevant article were based on different definitions. So we re-analyzed all gathered data by using a single standardized definition. An article of a large scaled multicenter study[43] that included our institute, where the data of the patients undergoing EPLBD with and without EST were calculated as one, was re-analyzed using its raw data in order to re-group both of them separately. Another limitation is that most articles included in this review are not prospective controlled studies, but retrospective studies. Therefore, further large-scale prospective randomized controlled studies will be needed not only to confirm our claims on effectiveness of EPLBD with or without EST, compared with both of EST alone and EPBD alone, but to assess the facts which affect the outcome and adverse events of EPLBD. In conclusion, recent accumulated results of EPLBD with or even without EST suggest that it is a safe and effective procedure for the removal of large or difficult bile duct stones without any additional risk of severe adverse events, when performed under appropriate guidelines.

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Intraductal papillary neoplasm of the bile duct

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Abstract

Intraductal papillary neoplasm of the bile duct (IPNB) is a variant of bile duct carcinoma that is characterized by intraductal growth and better outcomes compared with common cholangiocarcinoma. IPNBs are mainly found in patients from Far Eastern areas, where hepatolithiasis and clonorchiasis are endemic. According to the immunohistochemical profiles of the mucin core proteins, IPNBs are classified into four types: pancreaticobiliary, intestinal, gastric, and oncocytic. Approximately 40%-80% of IPNBs contain a component of invasive carcinoma or tubular or mucinous adenocarcinoma, suggesting that IPNB is a disease with high potential for malignancy. It is difficult to make an accurate preoperative diagnosis because of IPNB's low incidence and the lack of specificity in its clinical manifestation. The most common abnormal preoperative imaging findings of IPNB are intraductal masses and the involvement of bile duct dilation. Simultaneous proximal and distal bile duct dilation can be detected in some cases, which has diagnostic significance. Cholangiography and cholangioscopy are needed to confirm the pathology and demonstrate the extent of the lesions. However, pathologic diagnosis by biopsy cannot reflect the actual stage in many cases because different foci may be of different stages and because mixed pathologic findings may exist in the same lesion. Surgical resection is the major treatment. Systematic cholangioscopy with staged biopsies and frozen sections is recommended during resection to ensure that no minor tumors are left and that curative resection is achieved. Staging, histologic subtype, curative resection and lymph node metastasis are factors affecting long-term survival.

Key words: Intraductal neoplasm; Papillary cholangiocarcinoma; Biliary papillomatosis; Mucinous; Prognosis

Core tip: In this review, we have provided a more comprehensive understanding of "intraductal papillary neoplasm of the bile duct" than in other research articles. We found that preoperative pathologic diagnosis by biopsy could not reflect the actual stage in many cases because different foci might be of different stages and because mixed pathologic findings might exist in the same lesion. Staging, histologic subtype, curative resection and lymph node metastasis were factors affecting long-term survival.

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INTRODUCTION

In the biliary system there is a class of tumor that is characterized by predominant intraductal papillary growth, which can occur anywhere along the biliary tree. Growths are usually multifocal, with or without macroscopically visible mucin secretion, and can be of any type of pathological transformation, from low-grade dysplasia to invasive carcinoma. According to these features, these growths used to be identified by various names such as biliary papillomatosis, mucin-producing cholangiocarcinoma, mucin-hypersecreting bile duct tumor, and biliary intraductal papillary mucinous neoplasm. They share common phenotypic changes in tumorigenesis or tumor-progression and show more favorable prognoses compared with non-papillary cholangiocarcinoma. The clinical entity intraductal papillary neoplasm of the bile duct (IPNB) was added to the 2010 World Health Organization (WHO) classification, and it includes intraductal papillary cholangiocarcinoma and its precursor lesions[1]. Previous studies have usually focused on one or several aspects of this disease and have added to our knowledge about their clinicopathological features. This review aims to summarize this knowledge and provide a more comprehensive understanding of IPNB.

EPIDEMIOLOGY

IPNB is mainly found in patients in Far Eastern areas, such as Taiwan, Japan, and Korea, where hepatolithiasis and clonorchiasis are endemic. It is a rare disease, and papillary cholangiocarcinoma accounts for approximately 4%-38% of all bile duct adenocarcinomas. Current reports are of sporadic cases without a tendency to familial aggregation. IPNB most commonly develops in patients between 50 and 70 years of age and shows different sex preponderances in different regions such that the male-to-female ratio is nearly 1:2 in Taiwan, 1:1 in Japan, and 2:1 in Korea and Western countries[2-5].

ETIOLOGY AND PATHOGENESIS

Although the specific etiology and pathogenesis are unclear, IPNB is known to have two major risk factors: hepatolithiasis and clonorchiasis. Yeh *et al*[6] reported that nearly 87% of patients with IPNB had hepatolithiasis in Taiwan. Another study[7] from Korea suggested that 31% of IPNB patients had hepatolithiasis and 18% had clonorchiasis, but there was no such association in Western countries[2]. This phenomenon suggests that racial and environmental factors may play a role in the development of IPNB in addition to the two major risk factors.

The time lag between the development of hepatolithiasis and IPNB is 6-8 years, and intraductal carcinoma *in situ* can take 1-2 years to evolve into an invasive lesion. Considering the findings of mixed pathologic types in each case, as well as papillary adenocarcinoma in the background of papillary adenoma, a process of inflammatory cell-repair dysplasia and malignant transformation is likely[6,8,9] (Figure 1). Approximately 40%-80% of IPNBs contain a component of invasive carcinoma or tubular or mucinous adenocarcinoma, suggesting that IPNB is a disease with a high potential for malignancy[2,3,6,8,9]. Immunohistochemical study of surgically removed specimens shows that almost all IPNBs express CK7, CK20, and mucin (MUC)5AC, which are markers of biliary, intestinal, and gastric epithelium, respectively. This finding indicates that IPNB tumor cells retain a biliary immunophenotype and obtain intestinal and gastric immunophenotypes during carcinogenesis. MUC1 expression is frequently associated with the development of invasive lesions[2,4,6,10-14], especially tubular adenocarcinoma, while mucinous carcinoma is usually associated with negativity for MUC1 but positivity for MUC2. Sasaki *et al*[15] found that overexpression of enhancer of zeste homolog 2 might be associated with malignant behavior in IPNB, in parallel with up-regulated MUC1 expression and down-regulated MUC6 expression. Recently Nakanuma *et al*[16] provided evidence that peribiliary glands (PBGs) contain cells implicated in the origin of IPNB. Cardinale *et al*[17] suggested that IPNB might arise from biliary tree stem/progenitor cells (BTSCs) located in PBGs. In response to risk factors such as inflammation, BTSCs might undergo a series of genetic changes and progress from dysplasia to invasive carcinoma.

PATHOLOGY

IPNB usually appears as singular or multiple grayish-tan to yellow, friable, papillary masses anywhere along the biliary tree, and small lesions may at times be remote from the main tumor. Histologically, IPNB is defined by tumors that show papillary proliferation of neoplastic biliary epithelial cells with delicate fibrovascular stalks within the bile duct, the macroscopic or microscopic existence of mucin, and dilation of the proximal or remote bile duct. Hematoxylin and eosin staining and immunohistochemical profiling of the mucin core proteins are used to classify IPNB into four types[7](Table 1). The pancreaticobiliary type consists of columnar cells with eosinophilic cytoplasm and round nuclei. This type is often positive for MUC1 but negative for MUC2. The intestinal type resembles an intestinal villous neoplasm, and the neoplastic cells consistently express MUC2 and MUC5AC but not MUC1. The gastric type consists of columnar cells resembling gastric foveae that express MUC5AC but are negative for MUC1 and MUC2. The oncocytic type consists of cells with abundant, intensely eosinophilic cytoplasm that consistently express MUC5AC with focal expression of MUC1 and/or MUC2. The pancreaticobiliary type is the most common and is usually associated with invasive lesions, while the onco-

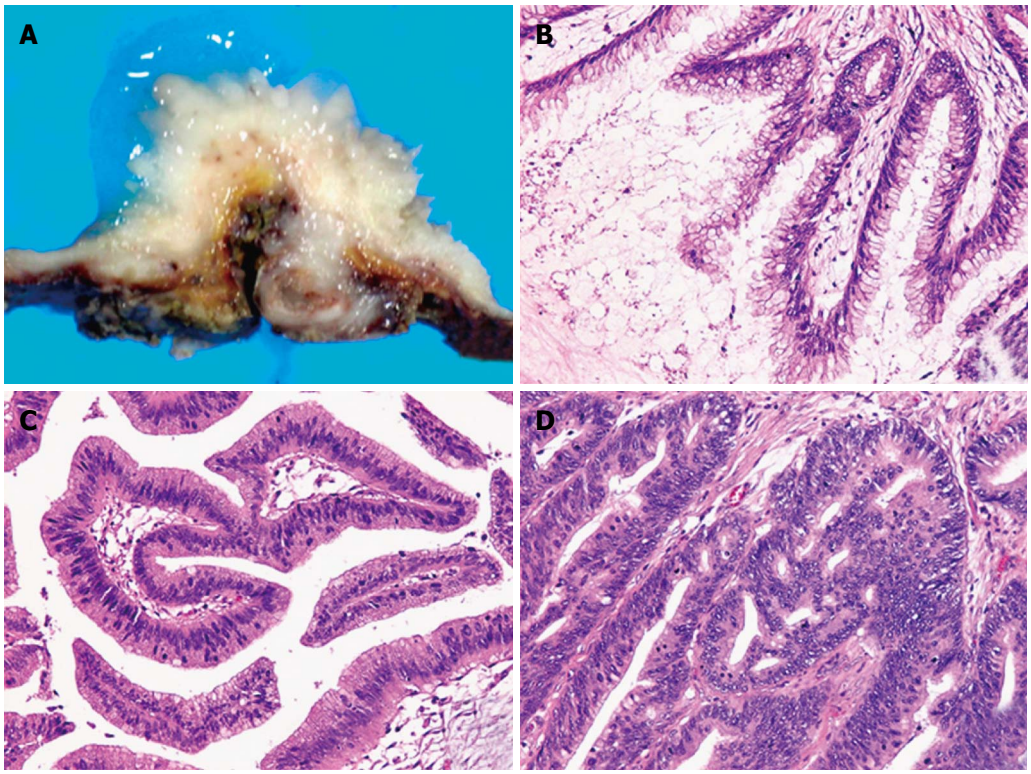

Figure 1 A representative case of intraductal papillary neoplasm of the bile duct with macroscopically visible mucin secretion. Within a single tumor (A), the coexistence of adenoma (B), borderline lesion (C), and adenocarcinoma (D) was found (hematoxylin and eosin stain, × 200).

Table 1 Histologic subtypes classified by mucin core protein and cytokeratins

Histologic subtype	Profile of MUCs			Cytokeratin	
	MUC1	MUC2	MUC5AC	CK7	CK20
Pancreaticobiliary	+	-	+	+	+
Intestinal	-	+	+	+	+
Gastric	-	-	+	+	+
Oncocytic	Focal+	Focal+	+	+	+

MUC: Mucin core proteins; CK: Cytokeratin.

cytic and gastric types are rare. According to the degree of dysplasia and depth of invasion, IPNB is classified into four stages: IPNB with low-to-intermediate grade dysplasia; IPNB with high-grade dysplasia; intraductal growth-type cholangiocarcinoma, AJCC stage T1; and intraductal growth-type cholangiocarcinoma, AJCC stage T2 or higher.

CLINICAL MANIFESTATION

The most common clinical manifestations of patients with IPNB are right hypochondralgia (35%-88.5%), repeated episodes of acute cholangitis (5%-59%), and obstructive jaundice (20%-36%). Anemia and loss of body weight are relatively less common. Some patients are asymptomatic[2,3,5-9]. Acute cholangitis, which is not a common presentation of conventional cholangiocarcinoma, is the second most common manifestation of IPNB. First, friable tumor emboli can easily detach from their origins, leading to acute obstruction of the bile duct. Second, more patients are diagnosed with biliary stones in IPNB than in typical cholangiocarcinoma. Third, macroscopic mucin hypersecretion can be observed in nearly one third of IPNB patients. Theoretically, abundant mucin discharge into the bile duct may intermittently impede bile flow, leading to obstructive jaundice and cholangitis, which can also cause volatile jaundice. The majority of IPNBs are found in the hilum and left liver; however, despite these variable locations, the primary site does not affect the course of the disease or prognosis[2].

LABORATORY TESTS

Laboratory data show common manifestations of obstruction of the bile duct such as elevation of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, direct bilirubin, gamma-glutamyl transpeptidas, alkaline phosphatase, *etc*. Yeh *et al*[18] found that an increased ALT level (> 36 U/L, $P = 0.022$) in IPNB was the only independent factor that could differentiate it from conventional cholangiocarcinoma. However, the specific relationship was not clearly elaborated, and this finding has not been supported by any other reports. Lee *et al*[8] observed elevation of CA19-9 in 20 of 50 IPNB patients, and the mean level was higher in patients with mucin hypersecretion. Yeh *et al*[6] found that an elevated serum CA19-9 level was detected in 35% of benign lesions, while 61% of malignant lesions

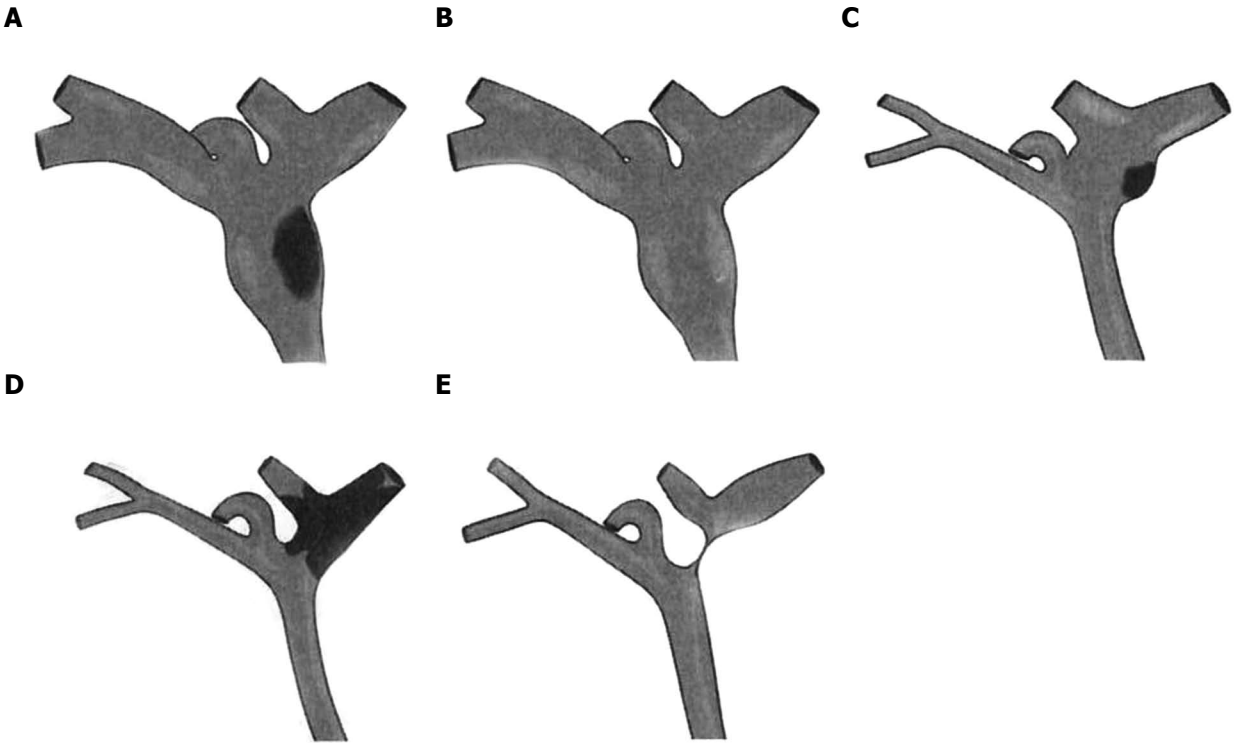

Figure 2 Schematic drawings of intraductal papillary neoplasms of the bile duct showing the five imaging patterns. A: Type 1: Diffuse duct ectasia with a grossly visible papillary mass; B: Type 2: Diffuse duct ectasia without a visible mass; C: Type 3: An intraductal polypoid mass within localized duct dilation; D: Type 4: Intraductal cast-like lesions; E: Type 5: A focal stricture-like lesion with mild proximal ductal dilation.

had elevated levels; however, there was no significant difference. Thus, the elevation of CA19-9 may be due to common cholestasis or cholangitis associated with mucin overproduction[19-22]. Additionally, an elevated CEA level was detected in nearly 25% of malignant IPNBs, so CEA may be of some value in differentiating intraductal papillary cholangiocarcinoma from its precursor lesions.

IMAGING FEATURES

The most common abnormal preoperative imaging findings for IPNB are intraductal masses and the involvement of bile duct dilation. Simultaneous proximal and distal bile duct dilation can be detected in some cases, which has diagnostic significance. Imaging patterns can be specifically classified into five subtypes[7,23] (Figure 2). Type 1 shows diffuse duct dilation with a grossly visible intraductal mass (45.4%). Type 2 shows diffuse and marked duct ectasia as in type 1 but without a grossly visible mass (23.7%). Type 3 shows an intraductal papillary mass with localized duct dilation (19.6%). Type 4 shows mild ductal dilation filled with intraductal cast-like lesions (4.1%). Type 5 shows a focal stricture-like lesion with mild proximal duct dilation (7.2%).

Ultrasound and ultrasonography

Ultrasound is sensitive for the detection of bile duct dilation, but it is only able to detect a low-echoic mass in nearly 41.2% of cases[8]. Although it helps to differentiate a stone from a tumor in most cases, the accuracy of ultrasound depends on the skill of the investigator. In addition, the presence of mucin cannot be detected on ultrasound because it is equally anechoic as bile. Endoscopic ultrasonography (EUS) and intraductal ultrasonography (IDUS) are useful for assessing the depth of invasion and involvement of the lymph nodes even in the presence of thick mucin, which is important to judge the resectability and predict prognosis. Therefore, it is difficult to distinguish between inflammatory wall thickness and the superficial spread of a tumor using EUS or IDUS[24,25].

Computed tomography and magnetic resonance image

Computed tomography (CT) can detect tumors larger than 1 cm and dilated bile ducts, and its sensitivity is 50%. The enhancement pattern of a tumor is related to the tumoral blood volume and blood flow as well as the prevalence of stromal space. IPNB is usually confined to the mucosa of the bile duct and suspended on small fibrovascular stalks, so it more often shows washout in enhancement scanning rather than the gradually persistent or progressive enhancement observed for conventional cholangiocarcinoma. IPNB appears as a slightly lower signal than hepatic parenchyma in T1WI and as a slightly higher signal in T2WI on magnetic resonance image (MRI) axial scanning. The enhancement pattern on MRI is similar to CT scan[26-28]. Neither CT nor MRI can detect the presence of mucin.

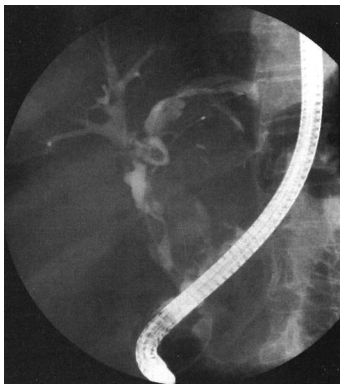

Figure 3 Endoscopic retrograde cholangiography showing an amorphous filling defect, suggesting the presence of mucobilia.

Table 2 Major utility of different imaging techniques

Techniques	Utility
Ultrasound	Detection of bile duct dilation Differentiation from a stone
EUS/IDUS	Assessing the depth of invasion and lymph node involvement
CT/MRI	Detect tumors larger than 1 cm and bile duct dilation Differentiation from conventional cholangiocarcinoma
Cholangiography	Define the extent of tumors Detection and drainage of mucin in ERC and PTC
Cholangioscopy	Confirm the histology and extent of lesions Adjuvant treatments
PET/CT	Detection of unsuspected distant metastases

EUS: Endoscopic ultrasonography; IDUS: Intraductal ultrasonography; ERC: Endoscopic retrograde cholangiography; PTC: Percutaneous transhepatic cholangiography; CT: Computed tomography; MRI: Magnetic resonance image; PET: Positron emission tomography.

Cholangiography

IPNB often involves the biliary epithelium either diffusely or multifocally, and the actual extent of the lesions usually exceeds CT, MRI and other conventional imaging findings. Cholangiography, including indirect (magnetic resonance cholangiography, MRC) and direct [endoscopic retrograde cholangiography (ERC), percutaneous transhepatic cholangiography (PTC)] cholangiography, is useful for showing the entire bile duct to define the extent of the IPNB[5,25]. MRC is noninvasive and can demonstrate the extent of narrowing or dilation of the bile duct and multifocal intraductal tumors, but it cannot detect the presence of overproduced mucin. ERC and PTC can show multiple small irregular filling defects in the bile duct wall. In patients with mucin overproduction, hypersecreted mucin draining through the ampulla and a patulous ampulla are the characteristic findings. On cholangiography, diffuse bile duct dilation and amorphous filling defects in the bile duct are characteristic[5] (Figure 3). However, a large amount of mucin secretion and obstruction by the tumor may prevent the complete opacification of the entire biliary system to locate the tumors. Cholangiography cannot detect multiple small tumors or lesions confined to the mucosa, and it cannot differentiate tumors from stones or benign strictures of the bile duct.

Cholangioscopy

Peroral cholangioscopy (POCS) and percutaneous transhepatic cholangioscopy (PTCS) can visualize the bile duct directly and confirm the histology and extent of the lesions to ensure that appropriate treatment is provided. In a study by Lee *et al*[8], PTCS revealed additional lesions in nearly one third of IPNB patients after radiologic imaging examinations including ERC and MRC. Therefore, preoperative cholangioscopy has been suggested to be essential. If the papillary orifice is dilated with or without mucin secretion, POCS can be performed immediately after ERC, resulting in an accurate early diagnosis of IPNB. This approach avoids the complications caused by PTCS, such as catheter dislodgement, hemobilia, and tumor seeding of the sinus tract[25,29].

Position-emission tomography/computed tomography

Malignant IPNB with a large mural nodule will present an increased fludeoxyglucose uptake. FDG PET has advantages in the detection of unsuspected distant metastases and in patients with renal dysfunction[24,30,31].

DIAGNOSIS AND DIFFERENTIAL DIAGNOSIS

It is difficult to make an accurate diagnosis preoperatively because of IPNB's low incidence and lack of a specific clinical manifestation. The combined application of different imaging techniques is very helpful (Table 2). Noninvasive imaging modalities such as CT and MRI usually fail to detect minor tumors and mucin; thus, cholangiography and cholangioscopy are needed to confirm pathology and demonstrate the extent of the lesions. Kang *et al*[32] reported that the accuracy of predicting macroscopic multiplicity based on preoperative radiologic imaging findings was 53.5%, with a false positive rate of 25.8% and a false-negative rate of 37.7%. In multifocal IPNB, different foci may be of different stages, and mixed pathologic findings may exist within the same lesion. This phenomenon suggests that pathologic diagnosis by biopsy cannot reflect the actual stage in many cases[8].

The differential diagnoses of IPNB includes recurrent pyogenic cholangitis with bile duct stones[31], mass-forming cholangiocarcinomas[18,27], and biliary mucinous cystic neoplasms (MCNs) (cystadenoma/cystadenocarcinoma)[33-36]. Both IPNB and recurrent pyogenic cholangitis with bile duct stones involve intermittent and incomplete biliary obstruction and intraluminal masses or filling defects on imaging. Mucin plugs or sloughed masses may be confused with stones. Invasive methods such as ERC or cholangioscopy may be necessary to differentiate these diseases. Mass-forming cholangiocarcinoma often appears as a single intrahepatic mass with upstream bile duct dilation and gradually persistent

or progressive enhancement on CT and MRI imaging. However, IPNB usually appears as multifocal papillary lesions with both upstream and downstream bile duct dilation with or without visible mucin overproduction that shows washout on enhancement scanning. The vast majority of MCN patients are female; 90% of cases are histologically benign but have the potential to recur and undergo malignant transformation. Multilocular cysts with separation or a cyst-in-cyst appearance are distinctive. Mucin produced by MCNs is confined and does not enter the biliary duct. Ovarian-like stroma is the characteristic microscopic finding. On the contrary, there is no such sex preponderance in IPNB, and 40%-80% of IPNBs are malignant. IPNBs communicate with the bile duct, and there is no ovarian-like stroma pathologically.

TREATMENTS

Surgical resection

Patients without distant metastasis are considered for surgical resection[37-39]. Preoperative IDUS or EUS is used for extrahepatic bile duct assessment and to look for the presence of lymph nodes. Cholangioscopy should be performed to determine the extent of the lesions and to draw up the optimal surgical strategy. During resection, systematic cholangioscopy is performed with staged biopsies and frozen sections. Patients with IPNBs localized to the intrahepatic bile duct are treated with hepatectomy. Patients with IPNBs involving one of the two intrahepatic bile ducts are treated with resection of the affected hemiliver and the common bile duct. For IPNBs localized to the extrapancreatic portion of the bile duct, complete resection of the bile duct from the biliary confluence to the intrapancreatic portion with extended lymphadenectomy is recommended. In cases of positive distal frozen sections, resection of the bile duct is performed with or without pancreatic resection (transduodenal resection). A partial liver resection can be performed when a proximal frozen section is positive in a single intrahepatic duct. Hilar lymphadenectomy has been suggested to be essential for tumors localized to the hilum or common bile duct, but a policy of selective application of caudate lobectomy and portal vein resection can be applied when it is necessary to achieve tumor clearance[40].

Liver transplantation

Surgical resection may remain incomplete due to the high risk of recurrence given positive margins in cases with superficial mucosal spread or recurrence on the remnant bile duct because of the high incidence of multifocal involvement. Resection of the entire biliary tree by liver transplantation and duodenopancreatectomy can be theoretically regarded as the only curative treatment. So far, case reports[41-44] on this approach indicate that patients with positive lymph nodes or major tumor invasion or associated severe comorbidities have not been eligible for liver transplantation. However, the preoperative assessment is usually insufficient for the majority of IPNBs. Thus, a strategy of initial resection to select patients without positive lymph nodes or advanced tumor invasion by definitive analysis of the specimen who would actually benefit from a subsequent liver transplantation seems to be reasonable[37].

Palliative treatment

In case major surgery is not indicated, palliative treatments are recommended[34]. Palliative intrahepatic tubing or percutaneous transhepatic biliary drainage can alleviate jaundice and cholangitis to prolong survival. Recently, new approaches such as percutaneous cholangioscopic laser ablation, cholangioscopic electrocoagulation, iridium-192 intraluminal therapy, and argon plasma coagulation are also useful for improved survival[45].

PROGNOSIS AND FOLLOW-UP

The prognosis of patients with IPNB has been consistently better than of those with conventional bile duct cholangiocarcinoma[40,46-49], and this finding may be related to the inherent biology of IPNB or its primarily intraductal growth pattern. Likewise, there is significant heterogeneity among these tumors. We summarize several factors affecting IPNB patient survival below.

Staging

There was no uniform staging system for IPNB before the 2010 WHO classification. However, it seems to be clear that the overall survival and recurrence-free survival of patients with IPNB is worse as one progresses from low-grade dysplasia to invasive carcinoma on the pathological scale[5,6]. Rocha *et al*[2] found that both depth of invasion and percentage of invasive carcinoma components correlated with survival (Figure 4). The depth of invasion, graded as $\geqslant$ 5 mm, < 5 mm, or none, was associated with survivals of 39, 128, and 144 mo, respectively ($P < 0.007$). In addition, the percentage of invasive carcinoma components, graded as $\geqslant$ 10%, < 10%, or none, was associated with survivals of 42 mo, 128 mo and 144 mo, respectively ($P < 0.03$).

Histologic subtypes

Kim *et al*[7] studied a group of 97 patients with IPNB and found that the histologic subtypes of IPNB were associated with different clinicopathologic features and prognoses. Specifically, the pancreaticobiliary type was distinct from the gastric and intestinal types with respect to higher histologic grades, more lymph node metastases, more postoperative recurrences, and worse clinical outcomes. The MUC1-high expressing group showed a shorter disease-specific and recurrence-free survival than the MUC1-low expressing group. In addition, patients with mucinous carcinoma showed a better prognosis compared with patients with tubular adenocarcinoma[3,13].

Curative resection

Lee *et al*[8] reported that the disease-free survival rate for patients who underwent curative resection was 81% at 5

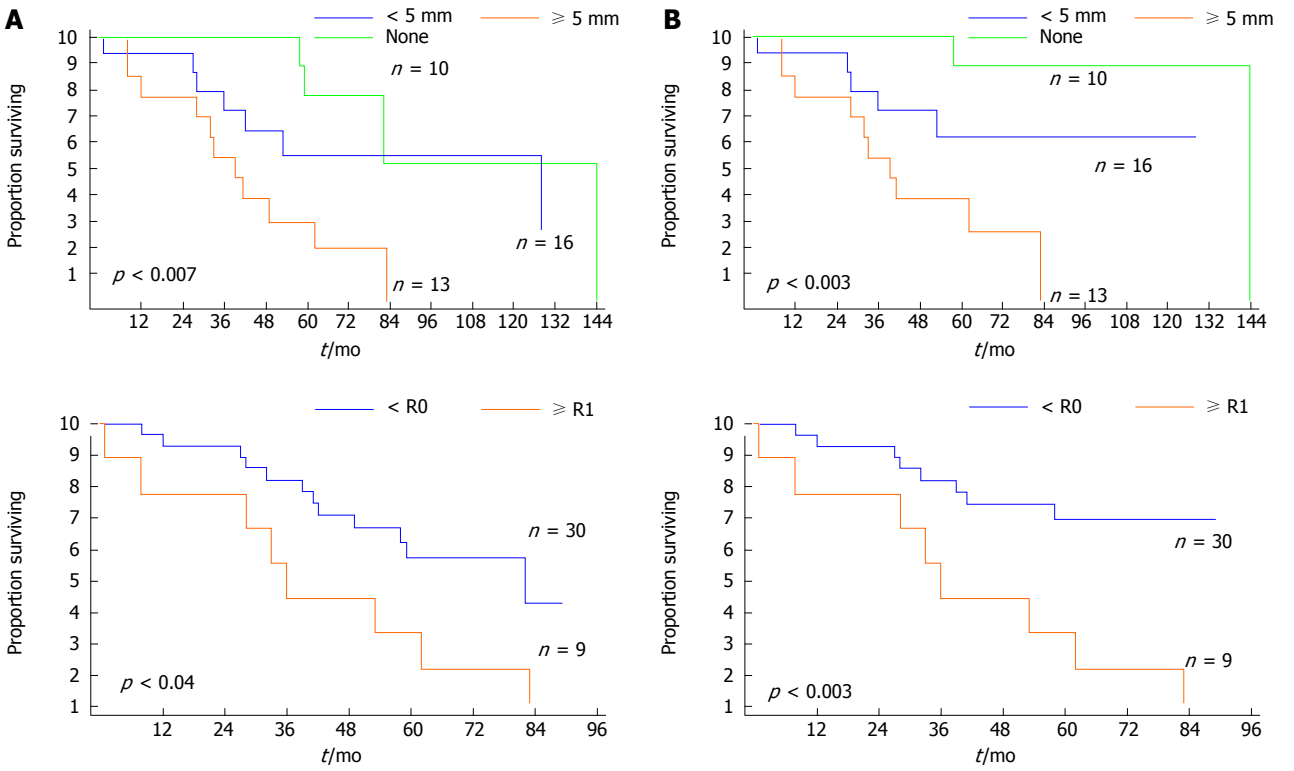

Figure 4 Kaplan-Meier survival estimates of overall survival (A) and disease-specific survival (B) according to the depth of extraductal invasion (none, < 5 mm, and ⩾ 5 mm) and resection type (R0 *vs* R1). $P < 0.05$ was considered significant.

years in a group of 58 cases of IPNB, and the mean survival period was 60.87 ± 5.86 mo (95%CI: 49.38-72.36), while it was 36.72 ± 6.61 mo (95%CI: 23.77-49.67) in patients who underwent palliative treatments such as percutaneous transhepatic biliary drainage (PTBD). Rocha *et al*[2] found that R0 resection was associated with an improved median survival time of 82 mo compared with 36 mo in the R1 resection group (Figure 4). Positive resection margins were strongly associated with shorter overall and recurrence-free survival rates, even for low-to-intermediate grade dysplasia[3].

Lymph node metastasis

Lymph node metastasis is rare in early-stage IPNB. Even in patients with invasive carcinoma, it is relatively less common than conventional cholangiocarcinoma. Yeh *et al*[5] found that the mean survival times with malignant IPNB with and without lymph node metastasis were 12.1 ± 5.1 mo (95%CI: 2.0-22.0) and 39.0 ± 6.7 mo (95%CI: 25.9-52.1), respectively.

A high rate of recurrence after surgical resection has been noticed. Incomplete preoperative assessment of the extent of IPNB might be an important contributing factor. Because small papillary tumors may not be detected by conventional radiologic methods, these undetected lesions, which are usually remote from the main tumor, may be the foci of recurrence[50,51]. In addition, positive margins related to the superficial spreading pattern of IPNB may be the reason for recurrence in many cases[8]. The recurrence rate at 5 years in benign IPNBs has been reported at nearly 20%, which rises to 60% in malignant cases, and most recurrences are locoregional[2,7]. Therefore, to improve the prognosis, full preoperative evaluation of the extent of disease is essential; and to detect recurrences, follow-up appointments scheduled every 3 mo for the first year and every 6 mo beginning in the second year are recommended. MRC is the optimal imaging method, while cholangioscopy can also be performed percutaneously or through the jejunal loop[34].

DISCUSSION

Recent studies[2,4,12,13,16,52] have revealed striking similarities between IPNB and pancreatic intraductal papillary mucinous neoplasm (IPMN). In both organs, these neoplasms arise within the duct system and show a predominantly intraductal growth pattern macroscopically and papillary proliferation with delicate fibrovascular cores and four types of tumor cells microscopically, occasional association with multiple lesions, possible progression to tubular adenocarcinoma and mucinous carcinoma, and more favorable biological behaviors and clinical outcomes. Based on these similarities, IPNB is recognized as a biliary counterpart of IPMN and can be differentiated from conventional cholangiocarcinoma. However, there are several differences between IPNB and IPMN. The most frequent phenotype is intestinal in IPMN but pancreaticobiliary in IPNB, which is more often associated with invasive carcinoma. The other important difference between IPNB and IPMN is with respect to mucin

hypersecretion. Mucin is macroscopically identifiable in most cases of IPMN but only in one third of IPNB cases. Considering the existence of goblet cells (one of the mucin-producing cells) and the expression of secretory-type mucin core proteins such as MUC2 and MUC5AC, this difference might be caused by the amount of mucin production.

Ohtsuka *et al*[9] separated IPNB with or without hypersecretion of mucin into two groups, and found that they were similar in terms of clinical features but somewhat different in pathological findings. IPNB without mucin hypersecretion showed a tubulopapillary growth pattern and uniform degree of cytoarchitectural atypia throughout the neoplasm, which was different from the mixed pathologic transformations in IPNB with mucin hypersecretion. Therefore, whether IPNB with and without mucin hypersecretion are different subtypes or they are distinct clinical entities needs further study.

In conclusion, intraductal papillary neoplasm of the bile duct is a rare biliary tumor with a better prognosis than conventional cholangiocarcinoma. Its specific mechanism of pathogenesis and progression has not yet been well defined[14,15,53-55], and its clinicopathologic features are similar to IPMN. Curative resection is the major treatment and an important favorable factor for long-term survival, especially in patients with early-stage IPNB.

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Cognitive-behavioral therapy for the management of irritable bowel syndrome

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Abstract

Irritable bowel syndrome (IBS) is a common disorder, reported to be found in 5%-20% of the general population. Its management accounts for up to 25% of a gastroenterologist's workload in the outpatient department, and the main symptoms are abdominal pain, bloating, and altered bowel habits. Despite a great amount of available pharmacological treatments aimed at a wide variety of gastrointestinal and brain targets, many patients have not shown adequate symptom relief. In recent years, there has been increasing evidence to suggest that psychological treatments, in particular cognitive-behavioral therapy (CBT), are effective for the management of IBS. This review discusses CBT for the management of IBS. CBT has proved to be effective in alleviating the physical and psychological symptoms of IBS and has thus been recommended as a treatment option for the syndrome.

Key words: Cognitive-behavioral therapy; Irritable bowel syndrome; Psychological treatment

Core tip: There is increasing evidence to suggest that cognitive-behavioral therapy (CBT) is effective for the management of irritable bowel syndrome (IBS). CBT can alleviate the physical and psychological symptoms of IBS, and has thus been recommended as a treatment option for the syndrome.

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INTRODUCTION

The prevalence of irritable bowel syndrome (IBS), a functional gastrointestinal (GI) disorder defined as discomfort or pain specifically associated with an abnormal bowel habit without structural or anatomical explanation, is reported to be between 5% and 20% in the general population[1], and its management accounts for up to 25% of a gastroenterologist's workload in the outpatient department[2]. IBS affects 10%-20% of the population in developed countries[3]. It also poses a huge burden to society due to direct and indirect costs, and reduced social functioning[4-6]. The cost of health care utilization and financial loss because of work absenteeism as a result of IBS is enormous in developed countries[5,7-9]. IBS is one of the most common diseases seen in primary care and specialty GI practices[10]. An estimated 12% of primary care patients and up to half of consultations in secondary gastroenterology practices are due to IBS-related symptoms[11,12]. It was observed by a tertiary care center that 38% of IBS patients had considered suicide because of their symptoms, highlighting the severe effect of IBS

on those patients[13]. Most patients with IBS suffer from coexistent mood disorder, anxiety, and neuroticism, and are reported to have a lower quality of life than other patients with serious chronic medical conditions such as diabetes mellitus or end-stage renal disease[14,15]. The diagnosis of IBS can be made on the basis of a series of symptoms fulfilling Rome Ⅲ criteria, but in clinical practice it is still frequently made by exclusion of an organic disorder after investigation[16]. There is a multifactorial etiology[17], altered pain perception, involvement of altered gut reactivity and motility, and alteration of the brain-gut axis in IBS[18]. Psychological and social factors can influence digestive function, symptom perception, illness behavior, and outcome[19]. Therefore, effective therapies for IBS are required in order to alleviate symptoms, and to reduce consultation behavior and consumption of other valuable medical resources.

Although pharmacological therapies can temporarily relieve symptoms, they are often costly and may result in negative side effects[20]. A substantial proportion of patients with IBS do not attain adequate relief through conventional medical approaches[21]. In recent years, there has been increasing evidence to suggest that psychological treatments, in particular cognitive-behavioral therapy (CBT), are effective for the management of IBS[22]. The cognitive-behavioral model was developed in the 1960s by the American psychiatrist and psychotherapist Rush *et al*[23], who applied it first to depression and then to anxiety disorders[24]. The model aims to identify patterns of thinking and behavior which deal with problems leading to negative emotions and hindering progress towards goals. When it is applied to physical health problems, it can reduce physical symptoms by addressing behavior patterns and physiological responses. There is excellent evidence for the efficacy of CBT in reducing symptoms in patients with IBS[25].

This review provides clinicians with an updated and predominantly evidence-based review of CBT for the management of IBS. Several systematic reviews and meta-analyses recently published in high impact factor journals and some randomized controlled trials are included. A better understanding of the recommended therapeutic approaches can lead to increased patient satisfaction, as well as reduced health-care costs.

CBT AND APPLICATION TO IBS

The idea that emotions can influence the sensorimotor function of the GI tract emerged at the beginning of the 19th century, and evidence from research conducted during that period is still valid[26]. Psychological disturbance, especially in referred patients, includes psychiatric disorders (*e.g.*, panic disorder, generalized anxiety disorder, mood disorder, and post-traumatic stress disorder), sleep disturbance, and dysfunctional coping[27]. A history of childhood abuse is common[19]. It has been indicated that up to two-thirds of patients with IBS in tertiary care centers have demonstrable psychiatric illness[28-30], and that these patients have a worse prognosis than those who are psychologically normal[31]. Approximately 50% of patients with a psychiatric disorder develop the condition before the onset of gastrointestinal symptoms, and psychiatric symptoms start at the same time in most of the remaining 50%[27]. Recently, it has been demonstrated that psychosocial factors, as an indication of the process of somatization, are independent risk markers for the development of IBS in a group of subjects previously free of IBS[32], and that the effect of psychosocial factors is strongest in severely affected IBS patients[33]. On the whole, IBS patients have been reported to have more psychological disturbance than control groups with organic gastrointestinal disease or general populations[24].

There is an increasing evidence for the effectiveness of CBT in alleviating the physical and psychological symptoms of IBS[2,25,34,35] and it has thus been recommended as a treatment option for the syndrome[17,19]. CBT has matured into a creative and rigorous synergy from empirical evidence and clinical innovation[36]. In the 1970s, a group of cognitive therapists in Philadelphia led by Aaron T Beck listened cautiously to what their clients were saying and turned to learning theory and the cognitive revolution to formulate a new theoretical account and therapeutic approach to depression[23]. CBT, from its inception growing out of basic and applied research[37], remains closely tied to ongoing research[38], and is used to deal with IBS. It was designed to educate participants about physical, cognitive, and behavioral factors which contribute to IBS; thus teaching them methods of enhancing self-control over stress, anxiety, and IBS symptoms; to correct dysfunctional thoughts and to prevent symptom relapse[39]. This is helpful for refractory IBS, as it blocks the vicious circle between psychological factors and symptoms. Thus, CBT that targets psychological disturbance may alleviate IBS symptoms[40].

COMPONENTS OF CBT FOR IBS

CBT is an extremely broad concept and the psychotherapy methods described in the literature have differed in their composition. However, each of the following components are generally included.

Education about IBS

IBS is presented as a functional bowel disorder, which is more ordinary than it appears, associated with bowel function, and as a distinct disorder with real physical symptoms, including abdominal pain, distress, anxiety, disruption to lifestyle, and embarrassment. Information is provided about intestinal function in general, such as the range of normal bowel frequency, the negative effects of straining to pass a motion or ignoring the urge, ways of dealing with constipation and diarrhea, pathogenesis, and treatment and clinical efficacy of IBS. IBS is considered a biopsychological disorder in which an association between life stress described as a normal part of life and an interaction between individuals and their environment,

and physiological changes leading to bowel irregularity is present. The impact of life stress on the gastrointestinal tract is characterized, with reference to the roles of central and autonomic nervous systems and the idea of "fight or flight" responses, including bowel muscle spasm[24]. The effect of psychological factors is discussed, which clarifies that pain signals from the site of physiological disturbance or damage passing through a special mechanism to the brain, which then interprets them by combining information from various stems. Abdominal pain is experienced, and this experience is influenced by current physiological arousal, focus of attention, mood, and beliefs about abdominal pain. For example, a patient who believes that eating food in a public place will always produce diarrhea symptoms might lead to an avoidance of social interactions, as well as anxiety when dining in a restaurant. The anxiety caused by this maladaptive thought may trigger diarrhea. The therapist aims to help the patient to recognize that a maladaptive idea adversely affects normal life functioning and symptom experience.

Good maintenance of a physician-patient relationship

Effectiveness of the therapy depends on maintaining a good relation between patients and medical personnel, forming a good working relationship. Experienced physicians know that maintaining a positive therapeutic physician-patient relationship for patients with IBS is of great importance; patients who experience this positive interaction with their physician have fewer IBS-related follow-up visits than patients who do not have this interaction[41]. Patients are encouraged to speak out about their own doubts and fears, and communicate with physicians; according to the patients' problems, physicians should be able to give a detailed answer in simple terms. In fact, most patients are conscious of the origin which has caused the symptoms of IBS, but the lack of proper cognitive meaning with symptoms is common. Patients are often organized to participate in discussions, and good experiences can be shared and improves their confidence in beating IBS. During the period, physicians and nurses can detect the patient's cognitive errors, correct them in an appropriate manner, and ensure smooth treatment progression.

Stress management

It is necessary for patients with IBS to understand that it is normal that the stress response appears when people meet stress. Identifying sources of stress for the individual concerned, working with them, and developing more helpful strategies for coping with them are prerequisite. Behavioral strategies made to ease the psychological pressure caused by cognitive behavioral efforts made in the face of stress. Positive behavior can mitigate stress and be beneficial to health, while a negative response will have the opposite effect.

Planning activities and training

An increased level of planning activity, including where and when certain foods should be eaten, also lifts mood and provides more distraction from the symptoms of IBS. Self-discipline training is an effective integrated relaxation technique, as there are some physiological changes in training in accordance with wishes.

Table 1 Randomized controlled trials reviewed by Khan *et al*[17]

Ref.	Country	Sample size	Psychological therapy used
Lackner *et al*[35]	United States	75	CBT
Lackner *et al*[42]	United States	71	CBT
Reme *et al*[43]	United States	149	CBT

CBT: Cognitive-behavioral therapy.

Table 2 Randomized controlled trials reviewed by Ford *et al*[2] (not including the trials described in Table 1)

Ref.	Country	Sample size	Psychological therapy used
Greene *et al*[45]	United States	20	CBT
Payne *et al*[46]	United States	22	CBT
Vollmer *et al*[48]	United States	34	CBT
Boyce *et al*[50]	Australia	105	CBT
Drossman *et al*[44]	United States	169	CBT
Tkachuk *et al*[47]	Canada	28	CBT
Kennedy *et al*[49]	England	149	CBT

CBT: Cognitive-behavioral therapy.

EVIDENCE FOR TREATMENT EFFICACY OF CBT

Khan *et al*[17] provide a useful review of the literature. Of the three controlled studies of patients with severe IBS, they noted that those in the CBT group showed reduced gastrointestinal symptoms and psychological distress to a greater extent than those in the control group. More details are given in Table 1.

A systematic review and meta-analysis carried out by Ford *et al*[2] was not included in this review. There were seven studies which compared CBT with control therapy or physicians' "usual management" in 491 patients[44-50]. IBS symptoms persisted in 118 of 279 individuals assigned to CBT, compared to 130 of 212 allocated to control therapy or physicians' "usual management". There was statistically significant heterogeneity and evidence among those studies, with small-sample studies showing no effect of CBT on IBS symptoms. When three studies conducted in the same center were excluded from the meta-analysis, the beneficial effect of CBT on IBS symptoms disappeared. More details are given in Table 2. Finally, they demonstrated that a range of different psychological therapies could significantly improve physical symptoms in IBS patients, with studies on CBT providing the greatest evidence.

For IBS, CBT has been studied more than any other form of psychological intervention in randomized controlled trials. In a recent review by Palsson *et al*[51], CBT outcomes for IBS treatment were compared with control

Table 3 Randomized controlled trials reviewed by Palsson *et al*[51] (not including the trials described in Tables 1 and 2)

Ref.	Country	Sample size	Psychological therapy used
Lynch *et al*[52]	Canada	21	CBT
Heymann-Monnikes *et al*[53]	Germany	20	CBT
Sanders *et al*[54]	United States	28	CBT
Hunt *et al*[55]	United States	54	CBT
Moss-Morris *et al*[56]	England	64	CBT
Craske *et al*[40]	United States	110	CBT
Ljotsson *et al*[57]	Sweden	195	CBT
Ljotsson *et al*[58]	Sweden	61	CBT
Oerlemans *et al*[59]	The Netherlands	75	CBT

CBT: Cognitive-behavioral therapy.

groups receiving usual medical care or on waiting lists for treatment, antidepressant or antispasmodic medication, placebo, or active psychological interventions such as supportive therapy, education, or stress management treatment. The substantial body of those studies demonstrates that CBT is an effective therapy for improving IBS. In the positive trials, gastrointestinal symptoms were almost uniformly found to be significantly ameliorated after treatment, sometimes substantially more than in control groups. Michelle *et al*[40] examined the efficacy of a CBT protocol for the treatment of IBS, which directly targeted visceral sensations. Participants (n = 110) were randomized to receive 10 sessions of either: (1) CBT with interoceptive exposure to visceral sensations (IE); (2) stress management (SM); or (3) an attention control (AC), and were evaluated at baseline, mid-treatment, post-treatment, and follow-up sessions. The results showed that the IE group outperformed AC on several indices of outcome, and outperformed SM in some domains. There was no difference observed between SM and AC. The results suggested that IE might be a particularly efficacious treatment for IBS. In spite of the fact that the majority of studies did not include any follow-up longer than 3 mo after medical treatment, there is some evidence that the therapeutic benefit of CBT for IBS can last 8 mo to 2 years after treatment termination. Apart from gastrointestinal symptom improvement, quality of life and emotional well-being are often documented to improve significantly from such therapy as well. More details are given in Table 3.

POTENTIAL PROBLEMS

Although CBT is considered the most well-studied psychological treatment for IBS[10], it is rarely available in routine care of IBS[60], and delivering the treatment may be cumbersome[12]. There is no evidence that patients' attributions for their illness and expectations/preferences for intervention influence the efficacy in any treatment. It is suggested that some patients do not understand the cognitive behavior model as applying to them and are thus unlikely to engage in CBT[61]. As this is a therapy which makes significant demands on patients' time, some will not feel able to make this commitment. Several problematic factors are a lack of trained therapists, high costs of delivering the treatment, and the practical difficulties for patients of scheduling weekly visits at a clinic[62,63]. Some modifications to the traditional CBT format have been evaluated by researchers, and these studies have demonstrated that CBT-based interventions can be delivered in different, and more cost-effective, formats[63,64]. Some clinicians have conducted studies investigating CBT for IBS where participants had a therapist contact *via* the internet (ICBT), defined as a web-based bibliotherapy with an online therapist contact. ICBT proved to be a promising cost-effective treatment modality for IBS, as it can be offered to IBS patients on a much larger scale than conventional psychological treatments[58,65]. Among gastroenterologists, development and testing of a CBT program for IBS has the potential to make it more widely available for IBS.

CONCLUSION

IBS is a prevalent chronic relapsing condition that is regularly associated with significant disability and has a considerable financial burden for the health service due to the consumption of resources including physician time, investigations, and costs of treatment[66]. The presence of clinically significant psychiatric symptoms in patients with IBS is an indication for psychotherapy, especially CBT. Although the availability of therapists who are trained in CBT and have specialist experience in IBS is limited, even when specialist referral is not an option, CBT has implications for gastroenterologists' own clinical practice. There is increasing evidence for the efficacy of CBT in alleviating the physical and psychological symptoms of IBS, and it has been recommended that it should be considered as a treatment option for the syndrome[28]. CBT is most appropriately offered to patients who have already had reasonable medical investigations but still have significant physical discomfort and psychological distress, and are interested in taking an active part in achieving greater control over their symptoms.

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ORIGINAL ARTICLE

Prognosis of patients with gastric cancer and solitary lymph node metastasis

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Abstract

AIM: To investigate the relationship of solitary lymph node metastasis (SLNM) and age with patient survival in gastric cancer (GC).

METHODS: The medical records databases of China's Beijing Cancer Hospital at the Peking University School of Oncology and Shanghai Tenth People's Hospital affiliated to Tongji University were searched retrospectively to identify patients with histologically proven GC and SLNM who underwent surgical resection between October 2003 and December 2012. Patients with distant metastasis or gastric stump carcinoma following resection for benign disease were excluded from the analysis. In total, 936 patients with GC + SLNM were selected for analysis and the recorded parameters of clinicopathological disease and follow-up (range: 13-2925 d) were collected. The Kaplan-Meier method was used to stratify patients by age (≤ 50 years-old, n = 198; 50-64 years-old, n = 321; ≥ 65 years-old, n = 446) and by metastatic lymph node ratio [MLR < 0.04 (1/25), n = 180; 0.04-0.06 (1/25-1/15), n = 687; ≥ 0.06 (1/15), n = 98] for 5-year survival analysis. The significance of intergroup differences between the survival curves was assessed by a log-rank test.

RESULTS: The 5-year survival rate of the entire GC + SLNM patient population was 49.9%. Stratification analysis showed significant differences in survival time (post-operative days) according to age: ≤ 50 years-old: 950.7 ± 79.0 *vs* 50-64 years-old: 1697.8 ± 65.9 *vs* ≥ 65 years-old: 1996.2 ± 57.6, all P < 0.05. In addition, younger age (≤ 50 years-old) correlated significantly with mean survival time (r = 0.367, P < 0.001). Stratification analysis also indicated an inverse relationship between increasing MLR and shorter survival time: < 0.04: 52.8% and 0.04-0.06: 51.1% *vs* ≥ 0.06: 40.5%, P < 0.05. The patients with the shortest survival times and rates were younger and had a high MLR (≥ 0.06): ≤ 50 years-old: 496.4 ± 133.0 and 0.0% *vs* 50-65 years-old: 1180.9 ± 201.8 and 21.4% *vs* ≥ 65 years-old: 1538.4 ± 72.4 and 37.3%, all P < 0.05. The same significant trend in shorter survival times and rates for younger patients was seen with the mid-range MLR group (0.04-0.06), but the difference between the two older groups was not significant. No significant differences were found between the age groups of patients with MLR < 0.04. Assessment of clinicopathological parameters identified age group, Borrmann type, histological type and tumor depth as the most important predictors of the survival rates and times observed for this study population.

CONCLUSION: GC patients below 51 years of age with MLR of SLNM above 0.06 have shorter life expec-

tancy than their older counterparts.

Key words: Gastric cancer; Solitary lymph node metastasis; Age; Metastatic lymph node ratio; Survival

Core tip: Among patients with gastric cancer and single lymph node metastasis, younger patients (≤ 50 years-old) tend to have less and shorter survival than their older counterparts; in particular, younger patients with the highest metastatic lymph node ratio have the worse prognosis.

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INTRODUCTION

Gastric carcinoma (GC) is one of the most commonly diagnosed cancers in China. The elderly (65 years and older) represent over one-half of these cases, and occurrence of GC in individuals younger than 40-years-old is relatively rare (approximately 5% of total reported cases)[1,2]. Studies of age-related GC progression and prognosis have yielded inconsistent findings. The collective data in the literature indicate both distinctly unfavorable outcomes for the younger patient population (citing more advanced disease at diagnosis and/or faster disease progression) and a seemingly paradoxical favorable overall survival (with early disease stage cases possibly providing a confounding subgroup effect); in addition, other studies have demonstrated a lack of age-related impact on GC prognosis[3-8].

Regardless of patient age, lymph node metastasis is a well-established critical prognostic factor and predictor of recurrence in GC. Histological detection of metastatic lymph nodes (MLNs) is strongly correlated with a high risk of recurrence, and this is an especially critical clinical finding for patients diagnosed in the early stages of GC to help design effectively robust, but safe, clinical management strategies[9-12]. While calculation of the metastatic lymph node ratio (MLR; single positive lymph nodes per total number of lymph nodes harvested) improves the sensitivity of predicting GC recurrence, minimizing the number of lymph node dissection is necessary to reduce the corresponding side effects, such as lymphedema[13-15].

A major clinical challenge in GC evaluation is determining the appropriate extent of lymph node dissection that is capable of detecting single lymph node metastasis (SLNM). The gastric lymphatic drainage system is particularly complex, and not all cases of SLNM are localized to the perigastric node area and are detectable by standard D2 lymphadenectomy. MLR, however, can help to overcome this limitation.

In this study, the differential prognostic features of younger and older GC patients with SLNM were investigated using MLR to gain further insights into the particular clinicopathological features and surgical outcomes of these two patient populations.

MATERIALS AND METHODS

Patients

The clinical records databases of two large metropolitan hospitals - Beijing Cancer Hospital of the Peking University School of Oncology, and the Shanghai Tenth People's Hospital Affiliated to Tongji University - were searched retrospectively to identify patients with histologically proven GC and SLNM who underwent resection surgery between October 2003 and December 2012. Patient records were selected for study inclusion according to the following eligibility criteria: available histological and pathological data related to diagnosis, including total number of resected lymph nodes. Of the 965 patients identified, 29 were excluded from analysis according to the presence of distant metastasis or development of gastric stump tumors following resection for benign disease.

In total, 936 patients with GC + SLNM were selected for analysis and the recorded parameters of clinicopathological disease and follow-up were collected. The patients were stratified by age (≤ 50, 50-64, and ≥ 65 years-old) and by MLR [< 0.04 (1/25) with > 25 lymph nodes sampled, 0.04-0.06 (1/25-1/15) with 15-25 lymph nodes sampled, and ≥ 0.06 (1/15) with ≥ 15 lymph nodes sampled].

Statistical analysis

All statistical analyses were carried out by the SPSS statistical software package, version 13.0 (SPSS Inc., Chicago, IL, United States). The Kaplan-Meier method, determined the overall and subgroups' 5-year survival rates, with the "event" endpoint being defined as death by any cause. The significance of differences between the various survival curves was assessed by a log-rank test. The chi-square test was used to evaluate differences between the clinicopathological disease and follow-up categorical variables. A P-value of < 0.05 was set as the threshold for statistical significance.

RESULTS

Characteristics of GC + SLNM patients

The study population's demographic and clinicopathological characteristics related to diagnosis and treatment, and stratified by age, are presented in Table 1. The median age was 68.6 years old (range: 26-86 years), with a relatively similar representation among the three age groups (20.5%, ≤ 50 years-old; 33.3%, 50-64 years-old; 46.2%, ≥ 65 years-old) but with a remarkably high proportion

Table 1 Characteristics of gastric cancer + solitary lymph node metastasis during clinical management, stratified by age *n* (%)

Parameter	Age (yr)			All patients (*n* = 965)
	≤ 50 (*n* = 198)	50-64 (*n* = 321)	≥ 65 (*n* = 446)	
Sex				
Male	117 (59.1)	207 (64.5)	272 (61.0)	596 (61.8)
Female	81 (40.9)	114 (35.5)	174 (39.0)	369 (38.2)
Tumor location				
Upper stomach	15 (7.6)	28 (8.7)	40 (9.0)	83 (8.6)
Middle stomach	79 (39.9)	131 (40.8)	183 (41.0)	393 (40.7)
Lower stomach	104 (52.5)	162 (50.5)	223 (50.0)	489 (50.7)
Gross type (Borrmann)				
Ⅰ	5 (2.5)	10 (3.1)	19 (4.3)	34 (3.5)
Ⅱ	51 (25.8)	59 (18.4)	103 (23.1)	213 (22.1)
Ⅲ	78 (39.4)	182 (56.7)	205 (45.9)	465 (48.2)
Ⅳ	64 (32.3)	70 (21.8)	119 (26.7)	253 (26.2)
Histological type				
High differentiation	1 (0.5)	2 (0.6)	4 (0.9)	7 (0.7)
Moderate differentiation	27 (13.6)	79 (24.6)	119 (26.7)	225 (23.3)
Low differentiation	170 (85.9)	240 (74.8)	323 (72.4)	733 (76.0)
Tumor status				
T1	16 (8.1)	29 (9.1)	42 (9.4)	87 (9.0)
T2	36 (18.2)	53 (16.5)	87 (19.5)	176 (18.2)
T3	75 (37.9)	125 (38.9)	194 (43.5)	394 (40.8)
T4	71 (35.8)	114 (35.5)	123 (27.6)	308 (32.0)
Metastasis lymph node ratio				
≥ 0.06	12 (6.1)	32 (10.0)	54 (12.1)	98 (10.2)
0.04-0.06	155 (78.3)	230 (71.7)	302 (67.7)	687 (71.2)
< 0.04	31 (15.6)	59 (18.3)	90 (20.2)	180 (18.6)
Postoperative chemotherapy				
No	4 (2.0)	11 (3.4)	29 (6.5)	43 (4.5)
Yes	194 (98.0)	310 (96.6)	417 (93.5)	922 (95.5)
Surgery				
Subtotal gastric resection	113 (57.1)	181 (56.4)	238 (53.4)	532 (55.1)
Total gastrectomy	85 (42.9)	140 (43.6)	208 (46.6)	433 (44.9)

in the mid MLR (0.04-0.06) group (71.2%). The median number of dissected lymph nodes was 24.3 (range: 5-71) and almost all patients received postoperative chemotherapy (with similar representation among the three age groups). The three age groups also showed statistically similar ($P > 0.05$) patient distribution for sex and tumor location, gross (Borrmann) type, histological (differentiation) type and status.

GC + SLNM patient outcome and predictors of survival

The study population's demographic and clinicopathological characteristics related to follow-up, stratified by age, are presented in Table 2. Twenty-nine of the patients were lost to follow-up and were excluded from further analysis. For the remaining overall study population, the median follow-up was 957 d (range: 13-2925 d) and the 5-year survival rate was 49.9%. Comparative analysis of the survival curves indicated significant differences among groups according to age (all three categories), Borrmann type (Ⅰ *vs* Ⅱ *vs* Ⅲ *vs* Ⅳ), histological type (high *vs* moderate *vs* low), and tumor depth (T1 *vs* T2 *vs* T3 *vs* T4) (all $P < 0.0001$); thus, all four of these variables were characterized as important predictors of survival.

Correlation of age with survival of GC + SLNM patients

Comparative analysis of the cumulative survival rates between the age categories (≤ 50 years-old: 950.7 ± 79.0, 50-64 years-old: 1697.8 ± 65.9, and ≥ 65 years-old: 1996.2 ± 57.6) showed statistically significant differences among all three (≤ 50 *vs* 50-64, $P < 0.001$; ≤ 50 *vs* ≥ 65 $P < 0.001$; 50-64 *vs* ≥ 65, $P = 0.020$) (Figure 1). The group of patients ≥ 65 years old had the best survival, and younger age (≤ 50 years-old) was found to correlate significantly with mean survival time ($r = 0.367$, $P < 0.001$) (Figure 2).

Correlation of MLR with survival of GC + SLNM patients

Comparative analysis of the cumulative survival rates between the MLR categories (< 0.04: 1527.0 ± 67.6, 0.04-0.06: 1851.1 ± 1527.0, and ≥ 0.06: 1352.1 ± 111.8) indicated that high MLR was associated with shorter survival (0.04-0.06 *vs* ≥ 0.06, $P = 0.030$; < 0.04 *vs* ≥ 0.06 $P = 0.028$). Comparison of the lower MLR categories showed no significant difference between the two (< 0.04 *vs* 0.04-0.06, $P = 0.681$). The high MRL group also showed a significantly lower 5-year survival rate than the other two groups (< 0.04: 52.8%, 0.04-0.06: 51.1%, and ≥ 0.06: 40.5%) (Figure 3).

Age-based stratification analysis of the MLR categories indicated that the younger patients with higher MLR had the shortest survival rate (Figure 4). In particular, the cumulative survival curves for patients with MLR of ≥

Table 2 Characteristics of gastric cancer + solitary lymph node metastasis during follow-up, stratified by age

Parameter	All patients (n = 936)	5-yr survival	P value
Age (yr)			< 0.0001
⩽ 50	188	29.10%	
50-64	311	55.00%	
⩾ 65	437	56.60%	
Borrmann type			< 0.0001
Ⅰ	33	92.40%	
Ⅱ	208	90.00%	
Ⅲ	455	40.40%	
Ⅳ	240	19.20%	
Histological type			< 0.0001
High differentiation	7	100.00%	
Moderate differentiation	215	74.60%	
Low differentiation	714	42.00%	
Tumor status			< 0.0001
T1	84	94.80%	
T2	170	82.10%	
T3	382	40.20%	
T4	300	30.40%	

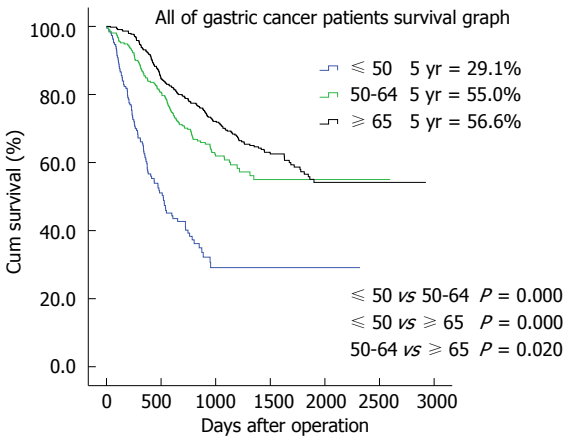

Figure 1 Cumulative survival of patients with gastric cancer + solitary lymph node metastasis according to age category.

0.06 (⩽ 50: 496.4 ± 133.0, 50-65: 1180.9 ± 201.8, and ⩾ 65: 1538.4 ± 72.4) were significantly different among the three age categories (⩽ 50 *vs* 50-65, P = 0.03; ⩽ 50 *vs* ⩾ 65, P = 0.000; 50-65 *vs* ⩾ 65, P = 0.005). The 5-year survival rates followed a similar trend: ⩽ 50, 0.0%; 50-65, 21.4%; > 65, 37.3% (Figure 4A). The cumulative survival curves for patients with MLR of 0.04-0.06 (⩽ 50: 847.3 ± 85.1, 50-65: 1410.1 ± 53.4, and ⩾ 65: 2140.7 ± 68.1) were also significantly different from the lowest age category (⩽ 50 *vs* 50-65, P = 0.000; ⩽ 50 *vs* ⩾ 65, P = 0.000); however, no difference was observed between the two older groups. The 5-year survival rates followed a similar trend: ⩽ 50, 23.9%; 50-65: 60.0%; ⩾ 65: 66.0%) (Figure 4B). The cumulative survival curves of patients with MLR of < 0.04 showed no significant differences among the age categories, and the 5-year survival rates were also similar (⩽ 50: 50.8%, 50-65: 56.3%, and ⩾ 65: 46.0%) (Figure 4C).

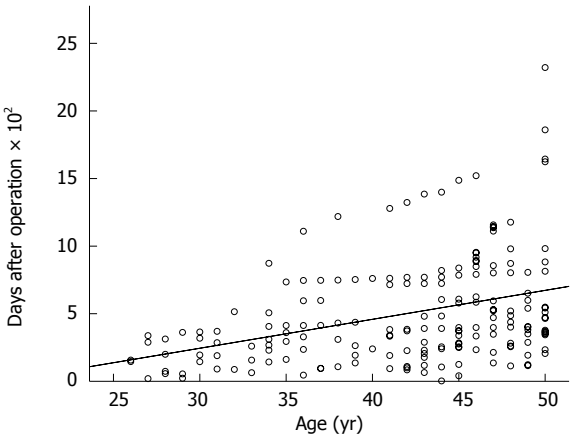

Figure 2 Correlation between age (⩽ 50 years-old) and mean survival days after surgery in patients with gastric cancer + solitary lymph node metastasis (r = 0.367; P < 0.001).

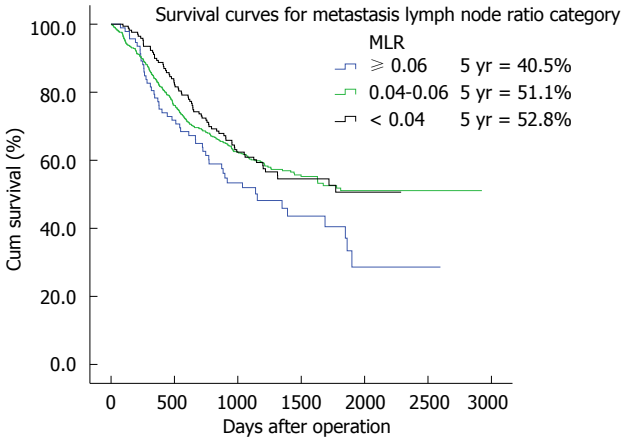

Figure 3 Survival curves for patients with gastric cancer + solitary lymph node metastasis according to metastatic lymph node ratio category. Survival is shown to be inversely associated with the ratio of positive nodes to lymph nodes harvested during surgery.

DISCUSSION

Some studies have indicated that a younger age of diagnosis may correspond to worse prognosis of GC[16]. From a histological perspective, GC in younger patients is more likely to be diffuse type, rather intestinal or mixed, and with disseminated morphology[17-19]. These features may underlie a more aggressive behavior of GC in this patient population or merely reflect a trend in diagnosis being made at a later disease state[4]; nevertheless, both of these issues are associated with poorer prognosis and may help to explain a key pathological difference between younger and older GC patients.

As cited in the Introduction, the collective research to date has yet to define the precise age-related epidemiological and clinicopathological characteristics of GC. For example, in a study of old and young GC patients matched by tumor stage, Moreira *et al*[20] found that younger age was associated with a more favorable out-

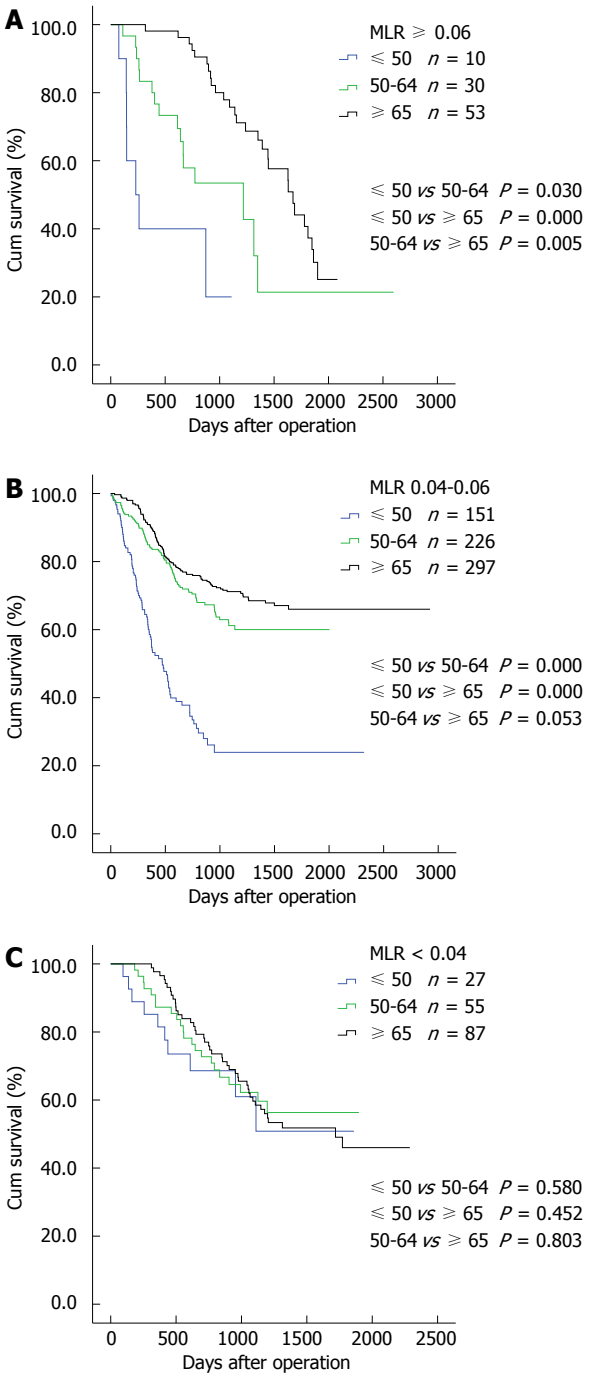

Figure 4 Cumulative survival of patients with gastric cancer + solitary lymph node metastasis and different metastatic lymph node ratio according to age category. A: $P < 0.05$ for comparisons among all three groups; B: Younger patients have significantly worse survival than the two older patient groups ($P < 0.01$); C: There are no significant differences among the three groups. Cumulative survival of patients with metastatic lymph node ratio (MLR) ⩾ 0.06 (A), 0.04-0.06 (B), and < 0.04 (C) by age.

come. Similarly, in a study of elderly and middle-aged GC patients matched for tumor extension, Kitamura *et al*[21] found that older age was associated with poorer overall survival and death within 30 d after surgery. Indeed, the increased risk of complications and death from surgery in general is well recognized, and a study of surgically-treated GC patients showed markedly better 5-year post-operative survival at all tumor stages[22].

It has been reported that younger (middle-aged) patients have better prognosis following curative resection of stage I tumors than their elderly counterparts[6]. Other reports, however, have demonstrated that younger age provides no benefit to survival when the GC is present in more advanced stages and that the most important prognostic factor in young patients is advanced nodal involvement[23-25]. Regardless of whether or not there is a distinctive malignant trait related to age, a key means towards improving survival rates is early diagnosis and timely application of curative resection.

The results from the current study confirmed the view that the relationship between GC prognosis and patient age is complicated. In general, the mean age of GC diagnosis falls within the 5th decade of life and cases younger than 50 years old are relatively rare[26,27]; however, by searching a large patient database we were able to analyze a patient population that equally represented young and old GC sufferers. The current study population showed a poorer prognosis for younger (⩽ 50 years old) patients, as evidenced by both cumulative and 5-year survival rates. Moreover, the younger patients had fewer surgery-related complications (data not shown) that may have benefited their recovery and prognosis.

Another important finding for the current study's population of patients with GC + SLNM was the relationship between age and mean survival days after surgery. The younger patients, who also had more aggressive tumors by histological analysis, survived for a significantly shorter duration following the resection treatment. This finding agrees with another study of GC patients that found diffuse cancers more likely to occur in younger patients and to be associated with poorer prognosis.

Depth of invasion and presence of MLNs are well-established and essential prognostic factors of GC[28], and nodal involvement is considered an especially significant clinical finding in early GC. Ten-year overall survival in node-positive patients has been reported at 27%, compared to the estimated 92% for node-negative patients[29]. Incomplete removal of MLNs, which harbor residual tumor cells, represents an increased risk of disease spread or recurrence. Indeed, studies of post-gastrectomy survival in GC patients have shown that survival rates improve in conjunction with number of lymph nodes removed for examination[30-32].

The benefit of lymphadenectomy was related to extent; however, it remains to be precisely determined. Both the Union for International Cancer Control and the American Joint Commission for Cancer have recommended that at least 15 lymph nodes be examined for correct assessment node metastatic status in GC (7th edition TNM system). Moreover, dissection of ⩾ 15 lymph nodes in resections with curative intent has been reported to significantly improve prognosis of patients with GC + MLN[33]. Yet, while the useful prognostic impact of this lower-limit criteria has been validated in several large clinical studies[34,35], no study to date has systematically

evaluated the risk to benefit ratio of precise numbers of lymph nodes for GC or its myriad of histological parameters.

The association of SLNM with depth of tumor invasion and its prognostic significance in GC are well established. Furthermore, it is generally accepted that GC patients with SLNM have a worse survival rate than those without SLNM (zero positive lymph nodes). The estimates of GC cases with single nodal metastasis distributed beyond the perigastric area range from 12.6%-29.0%, and it is hypothesized that this feature may be related to (caused by) complicated lymphatic drainage from the stomach[36-38]. However, a comparative study of patients with and without skip metastasis after standard D2 lymphadenectomy found no significant difference in survival between the two groups[39]. Sentinel node mapping with a visible tracer or radio-guided approach has limited accuracy in GC patients. Therefore, the current study evaluated the age-related 5-year survival rates of follow-up GC + SLNM patients using an array of clinicopathological parameters, and found that Borrmann type, histological type, and tumor status were significantly different among the groups and were related to patient survival.

MLR calculation is considered a simpler and (possibly) more effective method for prognosing patients with GC who undergo curative or radical resections[40-42], compared to the conventional lymph node staging systems. In particular, MLR could supplement the conventional N staging system when a limited number of lymph nodes are obtained, thus providing more accurate prognostic stratification in advanced GC[40,43-45]. Herein, as in some related previous studies, MLR was shown to be a better prognostic factor than the other clinicopathological parameters examined; however, no consensus has been made on the optimal categorization of MLR, as each study has used different standardization. In the current study, the GC + SLNM patients were categorized according to the number of harvested lymph nodes, and the data indicated that 5-year survival rates were associated with SLNM per lymph node harvested. Specifically, younger patients with MLR $\geqslant$ 0.06 and 0.06-0.04 had lower survival than older patients.

As discussed above, young adults may be more likely to present for diagnosis at an advanced disease stage. In the absence of an effective predictive marker, surveillance endoscopy of patients with positive family histories seems to be the only way to detect early stage GC. Such patients should also be educated on the signs and symptoms of GC, and more attention should be paid to younger patients with upper gastrointestinal symptoms, to improve their rate of early diagnosis. Multivariate analyses have indicated that younger patients undergoing curative resection have longer survival[46]. As D2 lymphadenectomy leads to the examination of more nodes, and improves prognostic accuracy in patients with or without MLNs[47], wider use of D2 lymphadenectomy may be essential for patients with GC, especially those of younger age.

In conclusion, among the GC + SLNM patients examined in this study, younger patients tended to have shorter survival than their older counterparts. In particular, younger patients with the highest MLR had the worst prognosis. Thus, the field should strive towards improving earlier detection rates for GC patients to help improve prognosis of these patients. For younger patients, who may be at greater risk of disease-related mortality but at less risk of surgery-related morbidities, D2 lymphadenectomy may be considered because it allows sampling of many more lymph nodes.

COMMENTS

Background

The current data on age-related differential prognoses for gastric cancer (GC) is inconsistent. This study was designed to investigate the potential age-related differences in survival of GC patients with solitary lymph node metastasis (SLNM) and to determine the clinical efficacy of metastatic lymph node ratio (MLR) as a more sensitive predictor of GC recurrence than traditional histopathological parameters.

Research frontiers

Among the patients with GC + SLNM examined in this study, the younger patients ($\leqslant$ 50-years-old) experienced greater mortality and shorter survival times than the older patients. In particular, the younger patients with the highest MLR had the worst prognosis.

Innovations and breakthroughs

Overall and 5-year survival rates of patients with GC and SLNM are lower in patients $\leqslant$ 50-years-old. Thus, efforts should be made to diagnose these cases earlier, and more sensitive intraoperative prognostic methods, such as calculating the MLR, should be applied. The younger age of these patients translates to their general better health and good candidacy for uncomplicated recovery from surgery, thus allowing for the realization of benefit from the more accurate surgical prognosis methods.

Applications

Strategies to improve earlier detection of GC should be devised and implemented, especially targeting the younger patient population. To achieve more accurate prognosis in this patient population, methods that allow for sampling of more lymph nodes, such as D2 lymphadenectomy, should be applied.

Terminology

Gastric carcinoma is usually detected at a later disease stage in younger individuals, and this fact may have cause a shorter survival time. It is generally accepted that GC patients with SLNM have a worse survival rate than those without SLNM. MLR is considered a simpler and more effective method for prognosing patients with GC who undergo curative or radical resections. D2 lymphadenectomy is considered when sampling many more lymph nodes.

Peer review

The authors thoroughly explained the problem of prognostic value of examined lymph node count in GC cases with SLNM, with respect to patient age. They have described, in detail, their data based on clinicopathological parameters and sufficiently discussed the implications of their findings in relation to the collective body of literature on this topic. The study showed that there is an age-related component to cumulative and 5-year survival of these cases, with younger age ($\leqslant$ 50-years-old) and higher MLR status being predictive of less and shorter survival.

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ORIGINAL ARTICLE

P115 promotes growth of gastric cancer through interaction with macrophage migration inhibitory factor

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Abstract

AIM: To investigate the role of P115 in the proliferation of gastric cancer cells and the mechanism involved.

METHODS: The RNA and protein level of P115 and macrophage migration inhibitory factor (MIF) in gastric cancer and normal gastric tissue/cells were measured and the effect of P115 on cell proliferation was assessed. The role of P115 in cell cycle checkpoints was investigated and the related proteins and signaling pathways, such as cyclin D1, Mcm2, p53, PCNA as well as the MAPK signaling pathway were determined. The interaction between P115 and MIF and the effect of P115 on MIF secretion were examined. The data were analyzed *via* one-way ANOVA comparisons between groups and $P < 0.05$ was considered significant.

RESULTS: P115 and MIF were both specifically expressed in gastric cancer tissues compared with normal gastric mucosa (both $P < 0.01$). The mRNA and protein levels of P115 and MIF in gastric cancer cell lines MKN-28 and BGC-823 were higher than in the human gastric epithelial cell line GES-1 (both $P < 0.01$). In MKN-28 and BGC-823 cell lines, P115 promoted cell proliferation and G_0-G_1 to S phase transition. In addition, several cell cycle-related regulators, including cyclin D1, Mcm2, PCNA, pERK1/2 and p53 were up-regulated by P115. Furthermore, the interaction between P115 and MIF was confirmed by co-immunoprecipitation assay. ELISA showed that P115 stimulated the secretion of MIF into the culture supernatant ($P < 0.01$) and the compensative expression of MIF in cells was observed by Western blotting.

CONCLUSION: P115 promotes proliferation of gastric cancer cells through an interaction with MIF.

Key words: Gastric cancer; P115; Migration inhibitory factor; Proliferation; Protein interaction

Core tip: Gastric cancer is one of the most common cancers. P115 is a tether protein which plays a key role in cell proliferation through combination with binding partners, including migration inhibitory factor (MIF). The present study showed that P115 and MIF were specifically expressed in gastric cancer tissues and cells. P115 promoted cell proliferation and G_0-G_1 to S phase transition. Cell cycle regulators, including cyclin D1, Mcm2, PCNA, pERK1/2 and p53 were up-regulated by P115. P115 interacted with MIF and stimulated the secretion of MIF into the culture supernatant. In summary, P115 promotes proliferation of gastric cancer cells through an interaction with MIF.

Li XJ, Luo Y, Yi YF. P115 promotes growth of gastric cancer through interaction with macrophage migration inhibitory factor. *World J Gastroenterol* 2013; 19(46): 8619-8629 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i46/8619.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i46.8619

INTRODUCTION

Gastric cancer is one of the most common cancers worldwide with a significant impact on human health[1]. Despite significant developments in the diagnosis and treatment of gastric cancer, the prognosis remains poor. Extensive surgery combined with chemotherapy is the most common therapy choice in the early stages of gastric cancer[2], while additional treatment options, such as gene therapy are desperately needed. With significant advances in genomics and proteomics, the discovery of a novel oncogene for therapeutic intervention remains a future challenge.

Cancer growth is a highly complex process involving alterations in gene expression and the interaction of many proteins. Golgi-vesicular transport protein P115 is a tether protein that plays an important role in many signal pathways required for cell proliferation[3] and has been extensively studied[4-6]. Macrophage migration inhibitory factor (MIF) was one of the first cytokines to be described and extensively studied[7]. More recently, MIF has been reported to be overexpressed in a number of cancers, including esophageal squamous cell carcinoma[8], glioblastoma[9], neuroblastoma[10], colonic cancer[11] and colorectal cancer[12]. The ability of MIF to promote tumor progression has been demonstrated and MIF has been shown to be a potential target for anti-cancer therapy. Hudson *et al*[13] and Jung *et al*[14] reported that MIF antagonized the activity of p53, which led to cancer progression. It was shown that the binding partner of MIF was JAB1/CSN5[15] which is known to be involved in the differentiation and morphogenesis of cells[16]. Furthermore, it is well known that upon binding to one of its receptors-CD74, MIF can increase the phosphorylation of Akt, ERK, MAPK and Stat3 which are all necessary for tumor proliferation.

Recently, a yeast two-hybrid interaction was examined to identify the intracellular proteins which might bind to MIF and mediate its secretion, and it was shown that P115 was a binding partner of MIF[17]. Previous research in our laboratory also demonstrated the same result. The objective of the present study was to evaluate the expression, the function in cell proliferation and the biological mechanism of P115 in gastric cancer.

MATERIALS AND METHODS

Cell culture

Human gastric cancer cell lines BGC-823 and MKN-28 were obtained from the American Type Culture Collection (Manassas, VA, United States). The human gastric epithelial cell line GES-1 was obtained from the cell bank of the Fourth Military Medical University. The cells were cultured in RPMI 1640 medium (Gibco, Maryland, United States) supplied with 10% FBS (Gibco, Maryland, United States), 100 units/mL penicillin and 100 μg/mL streptomycin at 37 ℃ in humidified 5% CO_2.

Immunohistochemistry

Thirty gastric cancer and 30 normal gastric mucosa specimens were obtained from the Department of Pathology, the First Affiliated Hospital, Chongqing Medical University from September 2008 to November 2009. Normal gastric mucosa specimens were obtained from normal tissues adjacent to the cancer tissue, and were pathologically confirmed as non-cancerous. The procedure was approved by the Ethics Committee. Samples were incubated with anti-P115 and anti-MIF rabbit polyclonal antibody (Cell Signaling Technology, United States) at 4 ℃ overnight, and then incubated with biotinylated goat anti-rabbit antibody (Santa Cruz Biotechnology, TX, United States) at room temperature for 15 min. DAB substrate was then used in the chromogenic reaction.

Construction of plasmids and transfection

The pCD-shRNA was reconstructed from pGPU6/GFP/Neo. Four shRNAs targeting P115 and shNC were designed as shown in Table 1. ShRNAs were ligated into the *BamH* I and *Bbs* I-digested pGPU6/GFP/Neo vector. The P115 expressing plasmid, pEGFP-N1-P115, was obtained from Jikai Company (Shanghai, China). Cells were seeded in 6-well plates and were transfected with 2 μg plasmids after reaching 70%-80% confluence using Lipofectamine 2000 (Invitrogen, Carlsbad, United States) following the manufacturer's instructions.

Reverse transcription-polymerase chain reaction and quantitative real-time polymerase chain reaction

RT-PCR was carried out using the AccessQuick™ One-Step reverse transcription-polymerase chain reaction [RT-PCR kit (Promega Co., Madison, United States)] according to the manufacturer's protocol. The oligonucleotide primers used were as follows: P115 sense: 5'-AACCTGGTGGCTGAACGGCAAG-3', P115 antisense: 5'-AGAAGCTTCACACCAGGCCAGC-3'. MIF sense: 5'-CGGGTTCCTCTCCGAGCTCACC3', MIF antisense: 5'-TGATGTAGACCCTGTCCGGGCTGA-3'. β-actin sense: 5'-GACCCAGATCATGTTTGAGACC-3', β-actin antisense: 5'-GCCAGGATAGAGCCACCAAT-3'. Total RNA was reverse transcribed to synthesize cDNA at 45 ℃ for 45 min. PCR was performed in a single reaction volume of 25 μL. The schedule consisted of incubation for 5 min at 94 ℃ followed by 30 cycles of 94 ℃ for 30 s, 56 ℃ for 45 s and 72 ℃ for 1 min, then incubation for 10 min at 72 ℃. The PCR products were subjected to 1.5% agarose gel electrophoresis. Quantitative real-time RT-PCR was performed using specific sense and antisense primers in a 25 μL reaction volume containing 12.5 μL of Absolute™ QPCR SYBR Green mix (Invitrogen), 0.25 pmol of each primer, and 0.5 μg of mRNA. Oligonucleotide primers were as follows: P115 sense: 5'-GGAGGGGAACAGTGATGGAG-3', P115 antisense: 5'-CAAAGCTGCTGCAATAACCC-3'. β-actin sense: 5'-CGGGAAATCGTGCGTGAC-3', β-actin antisense: 5'-TGGAAGGTGGACAGCGAGG-3'. The number of amplification cycles was 35, and the reaction were performed for 3 min at 50 ℃, 20 s at 95 ℃, and 30 s at 60 ℃, with an initial step at 95 ℃ for 3 min.

Table 1 Sequences of shRNA

shRNA name	Target site		Sequences
P115-shRNA1	1117 bp	S	5'-CACCGCAGCTTTGTACTATCCTAATTTCAAGAGA ATTAGGATAGTACAAAGCTGCTTTTTTG-3'
		A	5'-GATCCAAAAAAGCAGCTTTGTACTATCCTAAT TCTCTTGAAATTAGGATAGTACAAAGCTGC-3'
P115-shRNA2	1318 bp	S	5'-CACCGCGCTGTGCTGTTCTCTATTGTTCAAGAGA CAATAGAGAACAGCACAGCGCTTTTTTG-3'
		A	5'-GATCCAAAAAAGCGCTGTGCTGTTCTCTATTG TCTCTTGAACAATAGAGAACAGCACAGCGC-3'
P115-shRNA3	1578 bp	S	5'-CACCGCAACCCTCCAGTTTCTTTACTTCAAGAGA GTAAAGAAACTGGAGGGTTGCTTTTTTG-3'
		A	5'-GATCCAAAAAAGCAACCCTCCAGTTTCTTTAC TCTCTTGAAGTAAAGAAACTGGAGGGTTGC-3'
P115-shRNA4	1777 bp	S	5'-CACCGCAGTTGGTCCAAGGCTTATGTTCAAGAGACATAAGCCTTGGACCAACTGCTTTTTTG-3'
		A	5'-GATCCAAAAAAGCAGTTGGTCCAAGGCTTATG TCTCTTGAACATAAGCCTTGGACCAACTGC-3'
NC-shRNA	-	S	5'-CACCGTTCTCCGAACGTGTCACGTTTCAAGAGA ACGTGACACGTTCGGAGAACTTTTTTG-3'
		A	5'- GATCCAAAAAAGTTCTCCGAACGTGTCACGT TCTCTTGAAACGTGACACGTTCGGAGAAC-3'

Western blotting analysis

Cells were lysed in 100 μL RIPA lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 1% NP-40, 150 mmol/L NaCl, 1 mg/mL aprotinin, 1 mg/mL leupeptin, 1 mmol/L Na_3VO_4, 1 mmol/L NaF) at 4 ℃ for 30 min. Cell debris was removed by centrifugation at 12000 × *g* for 20 min at 4 ℃. Protein concentrations were determined by the Bradford assay. An equal amount of lysate (40 μg) was resolved by SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Millipore, Bedford, United States). The membranes were blocked with 5% nonfat milk at room temperature for 1 h and then incubated for 2 h with primary antibodies. The membranes were then incubated for 1 h with an appropriate horseradish peroxidase-linked secondary antibody (Santa Cruz Biotechnology, TX, United States). Antibodies to P115, MIF, cyclin D1, Mcm2, PCNA, p53 and β-actin were obtained from Cell Signaling Technology (MA, United States). Electrochemiluminescence was performed according to the manufacturer's instructions using a Bio-Rad imaging system. Quantity One software was used to quantify the density of bands.

Cell proliferation assay

Cells were seeded in 96-well plates at a density of 2000 cells/well and allowed to proliferate for 24 h, 48 h and 72 h. Cell proliferation ability was assessed by MTT assay. Briefly, MTT (5 mg/mL) was added to each well and the plate was incubated for a further 4 h before removal of the media. DMSO was then added to each well to solubilize the formazan crystals. The absorbance was read at a wavelength of 595 nm using a microtiter plate reader. All experiments were carried out in triplicate.

Flow cytometric analysis of cell cycle distribution

Flow cytometric analysis was performed as previously described[18]. Forty-eight hours after transfection, cells were harvested and fixed with 75% ethanol at -20 ℃ overnight. Cells were stained with propidium iodide (25 mg/mL) and RNaseA (200 mg/mL) at 37 ℃ for 30 min. The DNA content was analyzed using a FACScan flow cytometer (Beckman Coulter, Germany).

Co-immunoprecipitation of P115 and MIF

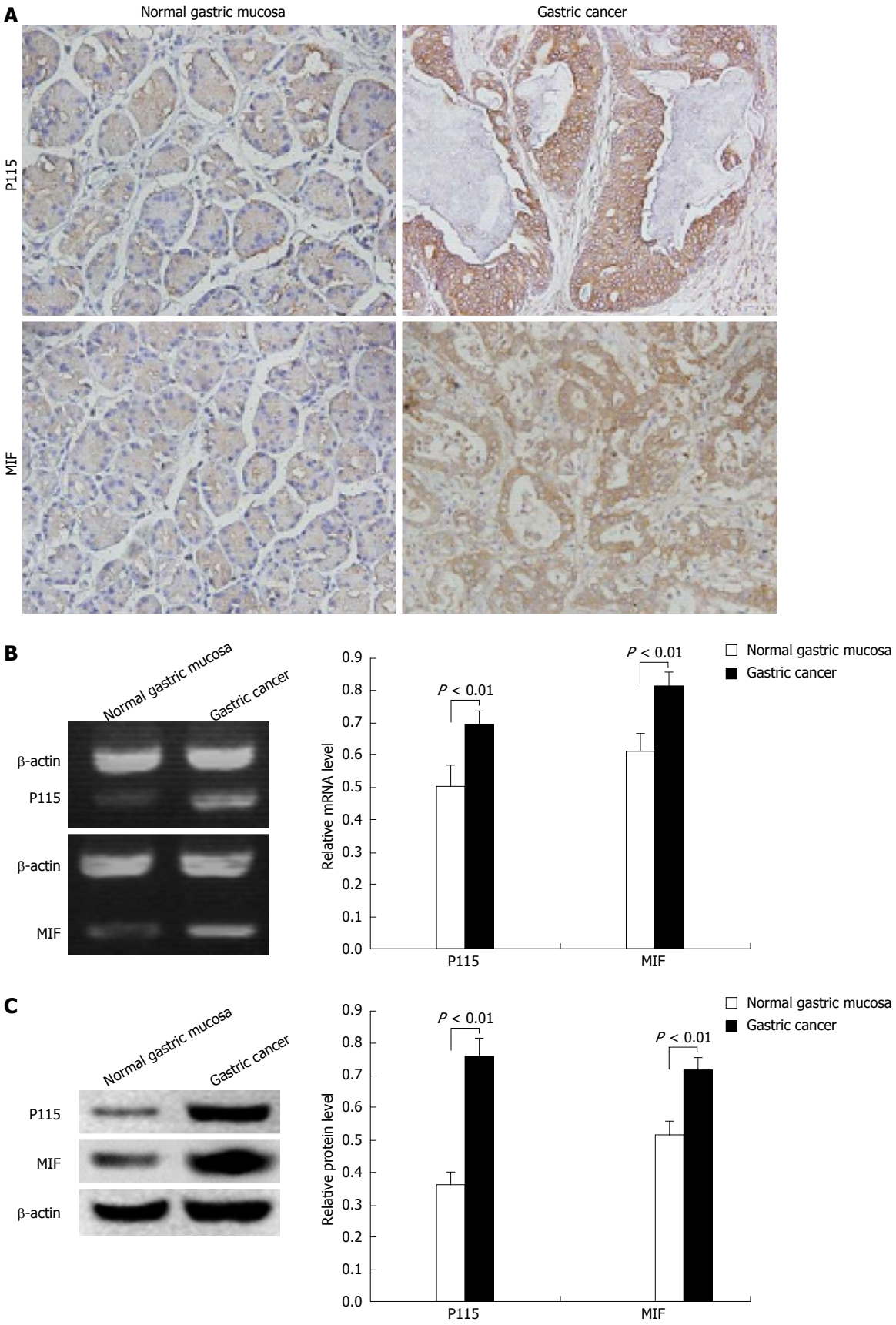
Cells were lysed in lysis buffer at 4 ℃ for 30 min. Cell debris was removed by centrifugation at 14000 × *g* for 5 min at 4 ℃. To remove non-specific binding, protein G sepharose beads containing mouse IgG were added to 200 μL protein and shaken slowly for 2 h at 4 ℃. The sample was then centrifuged at 2500 × *g* for 5 min at 4 ℃ and the supernatant was carefully removed for immunoprecipitation. 1 μg MIF antibody was incubated with the supernatant overnight and 42 μL protein G Sepharose beads were then added. The mixture was incubated for 3 h at 4 ℃ on a tube roller to precipitate protein complexes. The beads were obtained by centrifugation at 1000 × *g* for 60 s and washed twice with PBS. Finally, 20 μL loading buffer was added for SDS-polyacrylamide gel electrophoresis to assess P115.

ELISA assay

MIF level in the culture supernatant was determined by ELISA according to the manufacturer's recommendations. A polyclonal anti-MIF antibody was used as the capture antibody, and absorbance was measured at 450 nm in a microplate reader. The concentration of MIF in each sample was obtained by comparing absorbance values against the standard curve using r-MIF. Each experiment was performed in triplicate.

Statistical analysis

The data were expressed as mean ± SD of three independent experiments. The data were analyzed *via* one-way ANOVA comparisons between different groups with


A
Normal gastric mucosa
Gastric cancer
P115
MIF
B
Normal gastric mucosa
Gastric cancer
β-actin
P115
β-actin
MIF
Relative mRNA level
P < 0.01
P < 0.01
Normal gastric mucosa
Gastric cancer
P115
MIF
C
Normal gastric mucosa
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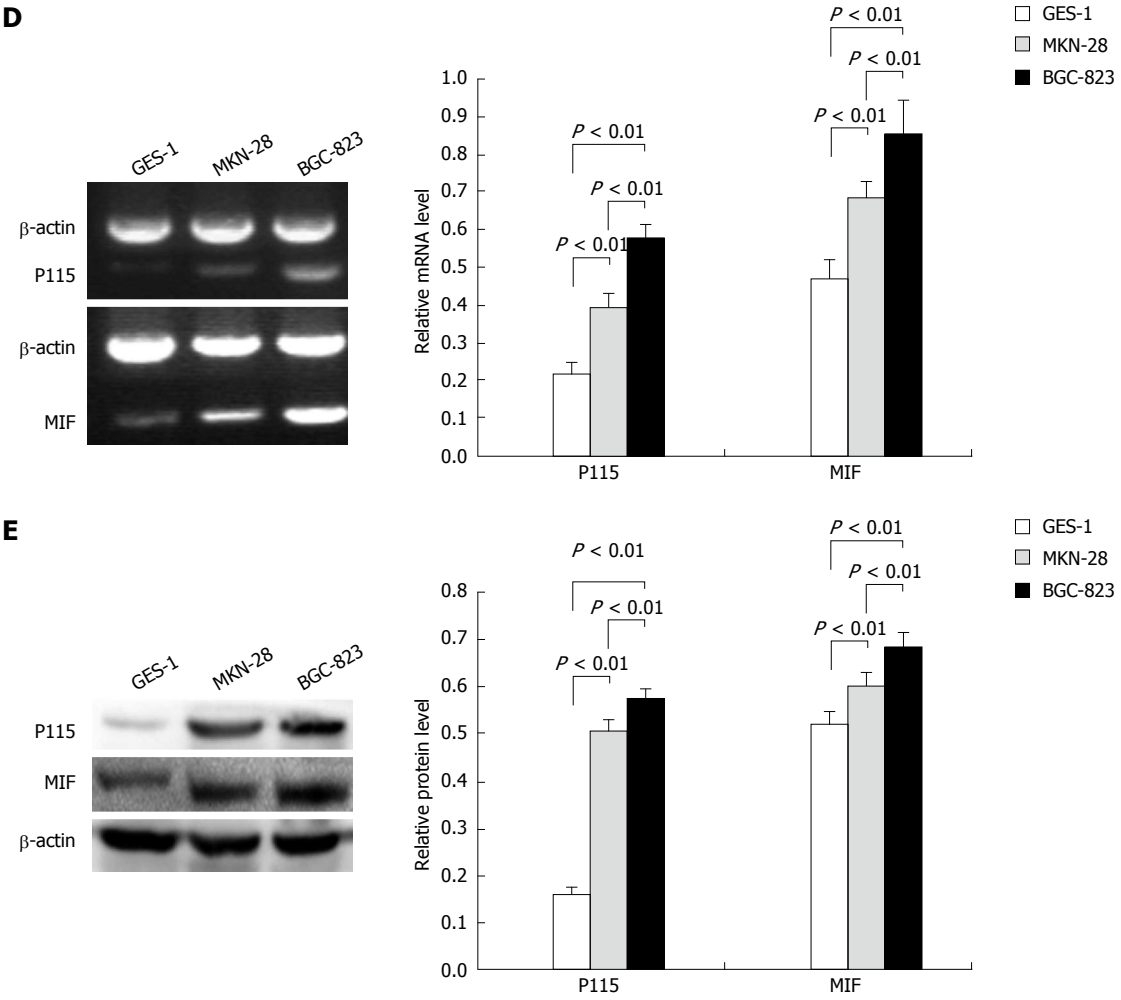

Figure 1 P115 and macrophage migration inhibitory factor were specifically expressed in gastric cancer. A: Immunohistochemistry showed that in gastric cancer tissue, P115 was expressed in Golgi and cytoplasm near the nucleus, and macrophage migration inhibitory factor (MIF) was expressed in cytoplasm and sparsely in membrane. In normal gastric mucosa tissue, P115 and MIF were negatively expressed (DAB stained, × 200). Real-time reverse transcription-polymerase chain reaction (RT-PCR) (B) and Western blotting (C) showed that mRNA and protein levels of P115 and MIF in gastric cancer tissue were higher than those in normal gastric mucosa tissue. RT-PCR (D) and Western blotting (E) showed that mRNA and protein levels of P115 and MIF in MKN-28 and BGC-823 cells were higher than those in the normal gastric mucosa epithelial cell line GES-1. β-actin was used as a loading control for RT-PCR and Western blotting. Data are mean ± SD of three experiments.

significance value set at $P < 0.05$.

RESULTS

P115 and MIF were specifically expressed in gastric cancer tissues

To examine whether P115 and MIF were specifically expressed in gastric cancer, the protein levels of P115 and MIF in human gastric tissue were first measured by immunohistochemistry (Figure 1A). It was shown that P115 was expressed in Golgi and cytoplasm near the nucleus, there were 12 positive samples (40.0%) in normal gastric mucosa and 22 positive samples (73.3%) in gastric cancer with 63.6% showing a strong positive rate (14 cases). MIF was expressed in cytoplasm and sparsely in membrane, there were 14 positive samples (46.7%) in normal gastric mucosa and 24 positive samples (80%) in gastric cancer with 66.7% showing a strong positive rate (16 cases).

Furthermore, the tissue homogenates of normal gastric mucosa and gastric cancer were lysed to measure P115 and MIF levels. Semi-quantitative RT-PCR analysis showed that P115 and MIF mRNA in gastric cancer (0. 694 ± 0. 046 and 0. 814 ± 0. 040, respectively, $n = 3$) were 1.377 and 1.326 times that in normal mucosa (0.504 ± 0.646 and 0.614 ± 0.054, respectively, $n = 3$) (Figure 1B, both $P < 0.01$). As shown in the semi-quantitative analysis of Western blotting results (Figure 1C), compared with normal gastric mucosa, the expression of P115 and MIF increased by 2.085- and 1.391-fold in gastric cancer (0.759 ± 0.058 *vs* 0.364 ± 0.037; 0.715 ± 0.040 *vs* 0.514 ± 0.044, respectively, $n = 3$; both $P < 0.01$).

P115 and MIF were specifically expressed in gastric cancer cell lines

P115 and MIF mRNA in different cell lines are shown in Figure 1D. P115 mRNA in the MKN-28 (0.391 ± 0.042, $n = 3$) and BGC-823 (0.513 ± 0. 038, $n = 3$) cell lines was 1.836- and 2.408-fold that in the normal gastric

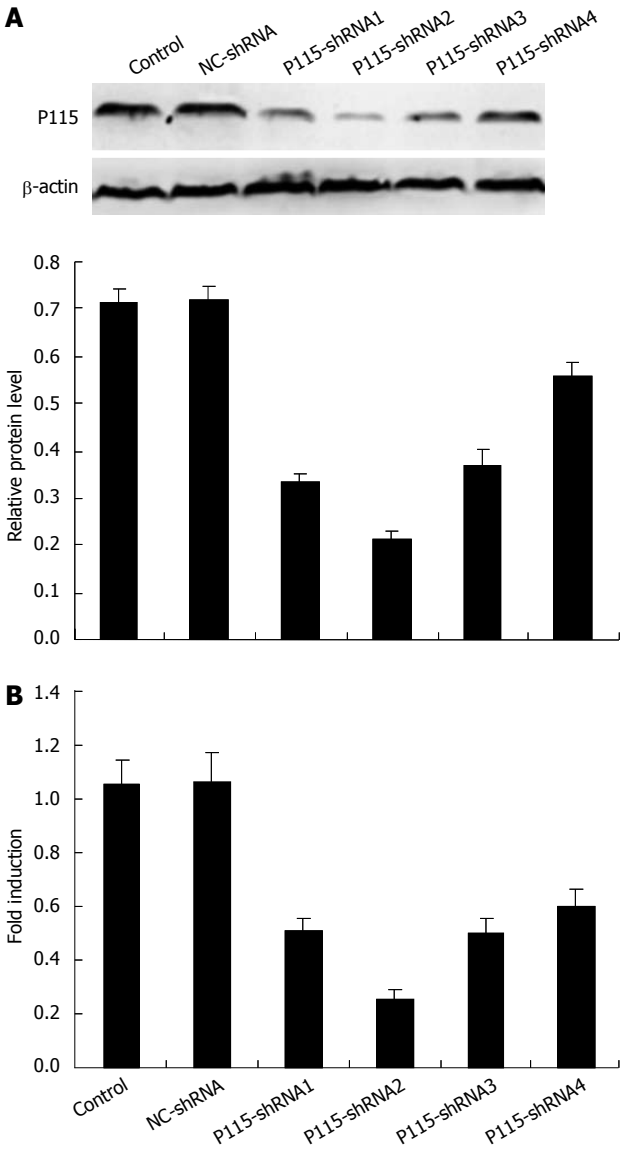

Figure 2 P115-shRNA plasmids reduced expression of P115 in BGC-823 cells. Cells were transfected with 2 μg P115-shRNA for 36 h and P115-shRNA2 was found to have the best silencing efficacy measured by Western blotting (A) and real-time polymerase chain reaction (PCR) (B). β-actin was used as a loading control for Western blotting and glyceraldehyde 3-phosphate dehydrogenase was used as an internal control for real-time PCR. Data are mean ± SD of three experiments.

mucosa epithelial cell line GES-1 (0.213 ± 0.036, $n = 3$), respectively (both $P < 0.01$). Moreover, in the poorly differentiated cell line, BGC-823, it was 1.312 times that in MKN-28 cells ($P < 0.01$). Correspondingly, MIF mRNA in MKN-28 (0.683 ± 0.046, $n = 3$) and BGC-823 (0.895 ± 0.104, $n = 3$) cells was 1.453 and 1.904 times that in GES-1 (0.470 ± 0.052, $n = 3$) and BGC-823 cells was 1.310 times that in MKN-28 cells (all $P < 0.01$).

Similar to the results of RT-PCR, the protein levels of P115 and MIF were markedly higher in MKN-28 and BGC-823 cells than in GES-1 cells (Figure 1E), and were higher in BGC-823 cells. Semi-quantitative analysis showed that the expression of P115 in MKN-28 (0.507 ± 0. 020, $n = 3$) and BGC-823 (0.547 ± 0. 015, $n = 3$) cells was 3.229- and 3.484-fold that in GES-1 cells (0.157

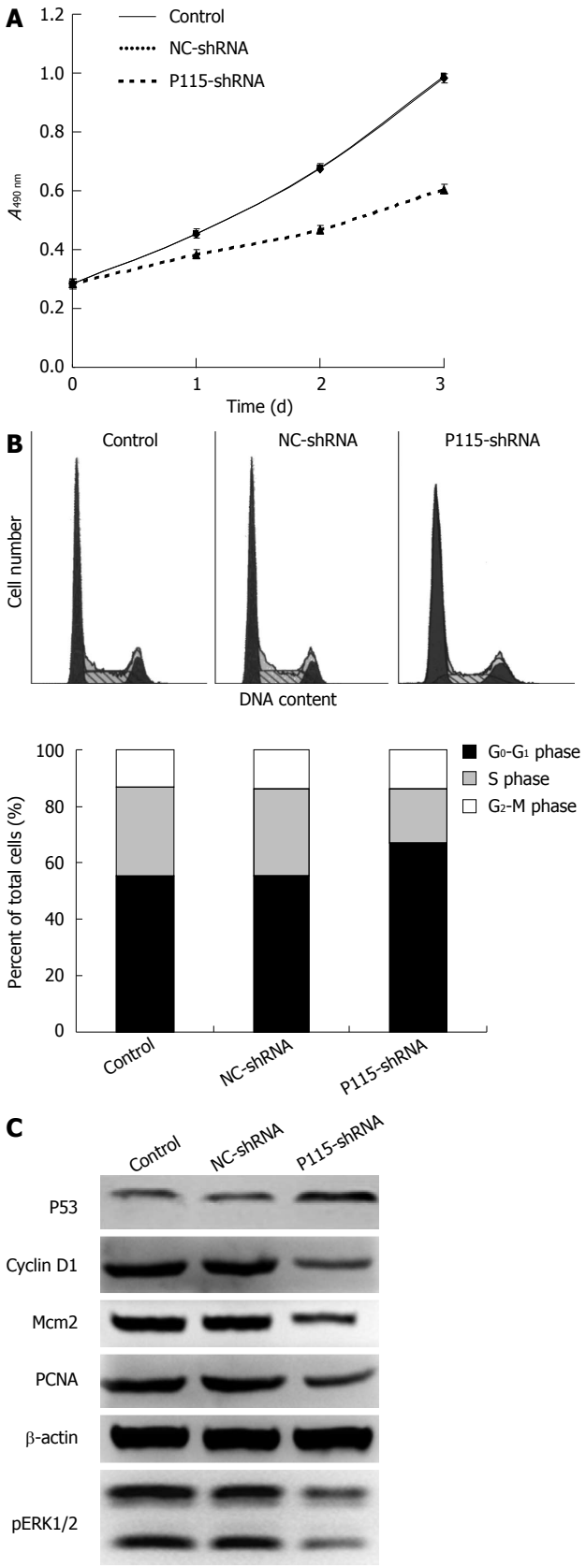

Figure 3 P115-shRNA inhibited cell proliferation and G_0-G_1 to S phase transition. A: After transfection with 2 μg P115-shRNA for 24, 48 and 72 h, the proliferation rate of BGC-823 cells was inhibited as detected by MTT assay; B: BGC-823 cells were transfected with 2 μg P115-shRNA for 48 h. The cell cycle was then measured by flow cytometry (B) and cell cycle regulators were measured by Western blotting (C). β-actin was used as a control for sample loading.

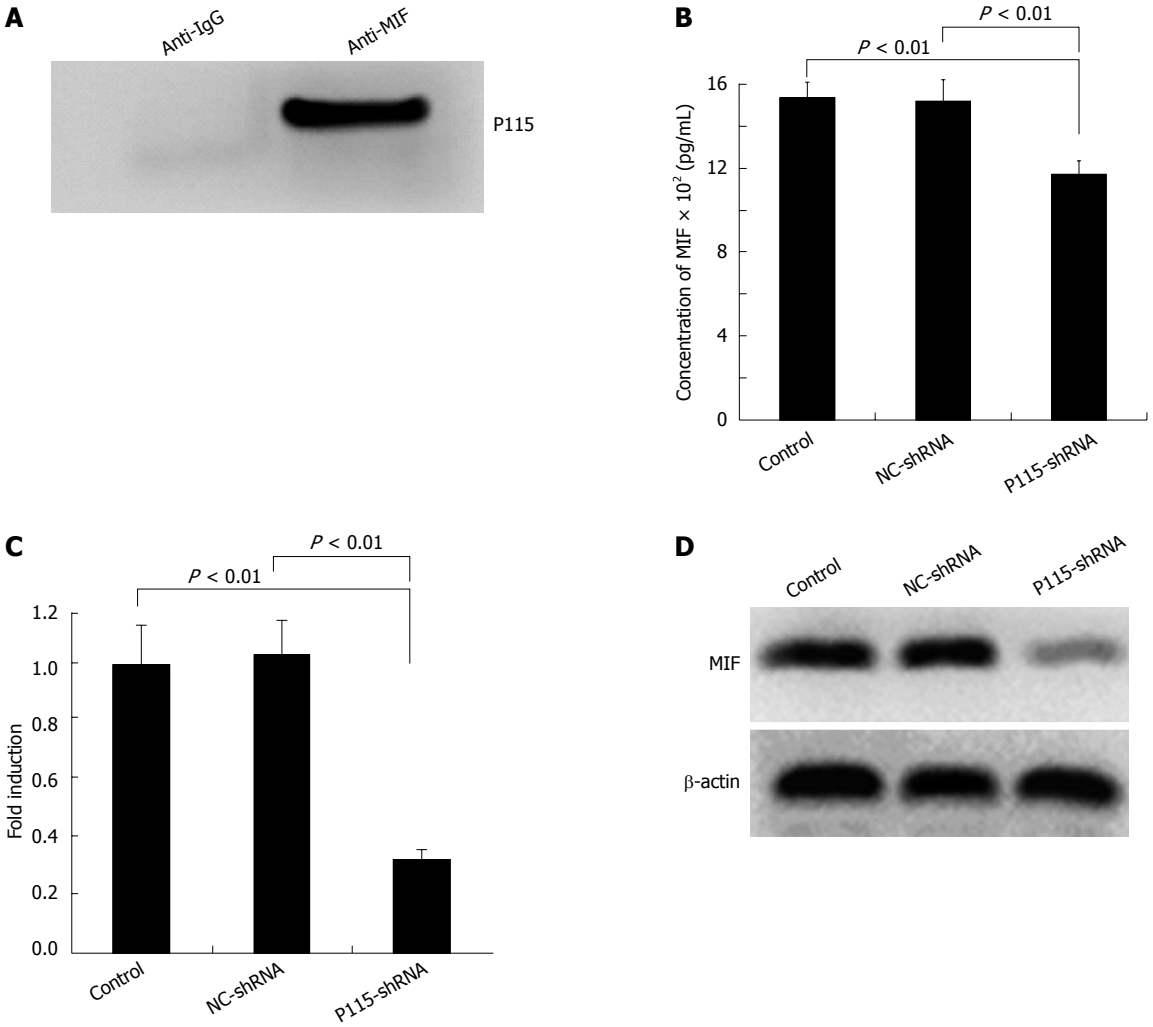

Figure 4 P115-shRNA inhibited the secretion and expression of macrophage migration inhibitory in culture supernatant and cells. A: Macrophage migration inhibitory factor (MIF) in BGC cells was extracted through protein precipitation with a multiple clone antibody, and P115 was detected in the protein complex by Western blotting; B: 48 h after transfection with 2 μg P115-shRNA, the secretion of MIF into the supernatant was inhibited as measured by ELISA. MIF in cells was reduced as measured by real-time PCR (C) and Western blotting (D). β-actin was used as a loading control for Western blotting and glyceraldehyde 3-phosphate dehydrogenase was used as an internal control for PCR. Data are mean ± SD of three experiments.

± 0.010, n = 3), and in BGC-823 it was 1.079-fold that in MKN-28 cells (all $P < 0.01$). The expression of MIF in MKN-28 (0.601 ± 0.017, n = 3) and BGC-823 (0.687 ± 0.015, n = 3) cells was 1.154- and 1.319-fold that in GES-1 cells (0.521 ± 0.020, n = 3), and in BGC-823 it was 1.143-fold that in MKN-28 cells (all $P < 0.01$).

P115-shRNA inhibited cell proliferation

To explore the biological function of P115, P115-shRNA plasmids expressing siRNA were constructed. First, the silencing efficiencies of 4 different P115-shRNAs were tested using Western blotting (Figure 2A) and real-time PCR (Figure 2B), which showed that the level of P115 was down-regulated most by 2 μg P115-shRNA2 plasmids (protein: 0.259 ± 0.034, n = 3; mRNA: 0.211 ± 0.010, n = 3) after 36 h transfection in BGC-823 cells (expression of P115 was relatively high) compared with control cells (protein: 0.727 ± 0.018, n = 3; mRNA: 1.041 ± 0.086, n = 3) and NC-shRNA (protein: 0.735 ± 0.010, n = 3; mRNA: 1.054 ± 0.094, n = 3), with silencing efficacy up to 76.8%. Therefore, P115-shRNA2 was selected for subsequent study. The proliferation rate of BGC-823 cells was then determined by MTT assay 24 h, 48 h and 72 h after transfection, and showed that the growth rate of P115-shRNA treated BGC-823 cells was obviously decreased (Figure 3A) compared with NC-shRNA.

P115-shRNA inhibited G_0-G_1 to S phase transition

The role of P115 in the cell cycle checkpoints was investigated (Figure 3B). FACS analysis revealed that P115-shRNA resulted in an 11.3% and 11.18% increase in cell number at G_0-G_1 phase compared with control and NC-shRNA in BGC-823 cells.

P115-shRNA inhibited expression of cyclin D1, Mcm2, PCNA, pERK1/2 and p53

As P115-shRNA caused cell cycle arrest, it was supposed that P115 could lead to a change in G_0-G_1 phase-related proteins and signaling pathways, such as cyclin D1, Mcm2, p53, PCNA as well as the MAPK signaling

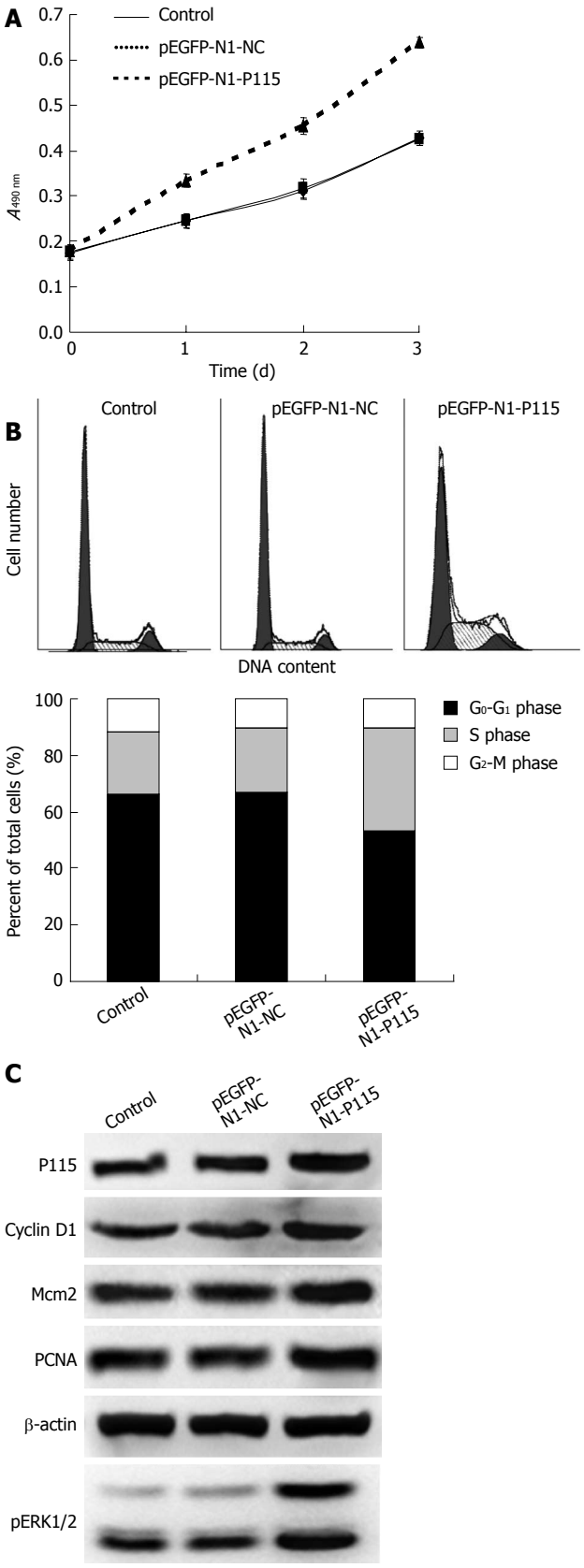

Figure 5 P115 promoted cell proliferation and G_0-G_1 to S phase transition. (A) After transfection with 2 μg pEGFP-N1-P115 for 24, 48 and 72 h, the proliferation rate of MKN-28 cells was increased as detected by MTT assay. (B) MKN-28 cells were transfected with 2 μg pEGFP-N1-P115 for 48 h. The cell cycle was then measured by flow cytometry (B) and cell cycle regulators were measured by Western blotting (C). β-actin was used as a control for sample loading. Data are mean ± SD of three experiments.

pathway. Therefore, the above proteins and phosphorylation of ERK1/2 were assessed. It was shown that cyclin D1, Mcm2, PCNA and pERK1/2 were significantly decreased by P115-shRNA in BGC-823 cells, which explained the effect of P115 on cell cycle phase. In addition, p53 was up-regulated by P115-shRNA (Figure 3C).

Interaction between P115 and MIF was detected by co-immunoprecipitation assay

MIF in BGC-823 cells was extracted through protein precipitation with a multiple clone antibody and the protein complex was detected by Western blotting. As shown in Figure 4A, P115 was detected in the protein complex, indicating that there was an interaction between MIF and P115 protein.

P115-shRNA inhibited expression of MIF in cells and secretion of MIF into supernatant

Considering that MIF is a secretory protein, the level of MIF in the culture supernatant was assessed by ELISA. This assay showed that the secreted concentration of MIF in the culture supernatant in P115-shRNA treated BGC-823 cells was markedly reduced (1173.67 ± 63.47 pg/mL, n = 3) compared with control (1535.62 ± 77.25 pg/mL, n = 3) and NC-shRNA treated cells (1517.69 ± 102.51 pg/mL, n = 3), this difference was statistically significant (P < 0.01, Figure 4B). In addition, MIF mRNA and protein in cells were also detected. As shown in Figure 4C, P115-shRNA decreased the level of MIF mRNA in BGC-823 cells, and Western blotting showed the same trend as real-time PCR, in that P115-shRNA decreased the expression of MIF (Figure 4D).

pEGFP-N1-P115 promoted cell proliferation, G_0-G_1 to S phase transition, expression of G_0-G_1 phase-related proteins and secretion of MIF into supernatant

The role of P115 in gastric cancer cells was assessed from another point of view. It was shown that after transfection with 2 μg pEGFP-N1-P115 for 24, 48 and 72 h, the proliferation rate of MKN-28 cells (expression of P115 was relatively low) was markedly increased (Figure 5A) and the transition of G_0-G_1 phase to S phase in MKN-28 cells was accelerated by 13.71% and 13.9%, respectively, compared with control and pEGFP-N1-NC (Figure 5B), suggesting that stimulation of cell growth by P115 was associated with the distribution of cell cycle phase. Correspondingly, cyclin D1, Mcm2, PCNA and pERK1/2 were significantly increased by pEGFP-N1-P115 in MKN-28 cells (Figure 5C).

ELISA showed that the secreted concentration of MIF in the culture supernatant in pEGFP-N1-P115 treated MKN-28 cells was markedly increased (1696.38 ± 107.95 pg/mL, n = 3) compared with control (1227.64 ± 90.58 pg/mL, n = 3) and pEGFP-N1-NC treated cells (1208.63 ± 101.78 pg/mL, n = 3), and the difference was statistically significant (P < 0.01, Figure 6A). As shown in Figure 6B, MIF mRNA in pEGFP-N1-P115 treated cells was increased and Western blotting also indicated that

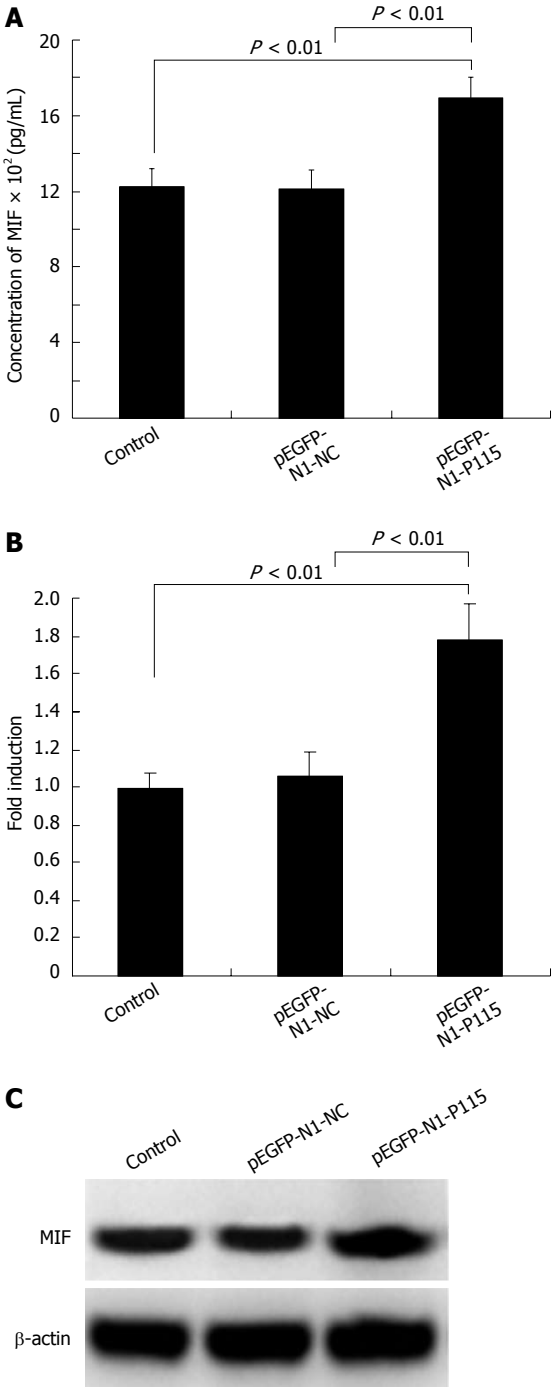

Figure 6 P115 promoted the secretion and expression of MIF in culture supernatant and cells. (A) 48 h after transfection with 2 μg pEGFP-N1-P115, the secretion of migration inhibitory factor (MIF) into the supernatant was measured by ELISA. MIF in cells was increased by pEGFP-N1-P115 as measured by real-time polymerase chain reaction (PCR) (B) and Western blotting (C). β-actin was used as a loading control for Western blotting and glyceraldehyde 3-phosphate dehydrogenase was used as an internal control for PCR. Data are mean ± SD of three experiments.

pEGFP-N1-P115 increased the expression of MIF (Figure 6C).

DISCUSSION

The complicated molecular mechanisms of carcinogenesis and the interaction of multiple oncogenes in gastric cancer challenge our ability to identify novel and rational molecular therapeutic targets. The present study demonstrates that P115 may be a potential tumor biomarker and therapeutic target which is overexpressed in human gastric cancer. Interaction with MIF may be involved in its molecular mechanism.

P115 has been demonstrated to be involved in intra-Golgi transport[6] and can bind to the Golgi-associated proteins, GM130[19] and giantin[20], which both play an important role in mitosis, that is, P115 is essential for biogenesis of the Golgi apparatus[21,22]. MIF is a secretary protein which plays an important upstream role in the regulation of diverse cellular responses[23-25]. The role of MIF has been emphasized by the finding that high expression of MIF is associated with the incidence or the severity of oncologic diseases[26-28]. The data from this study showed that overexpression of P115 significantly enhanced the secretion of MIF, which indicated that P115 might be one of the stimuli inducing MIF secretion through direct interaction. Merk *et al*[29] reported that MIF was co-secreted with P115, indicating that P115 had a specific role in MIF export, which is consistent with our results. MIF lacks a signal sequence and is secreted by an unconventional route for protein export. Stimuli induce the rapid release of MIF from preformed and cytoplasmic pools, which is followed by an upregulation of MIF mRNA expression and a replenishment of intracellular protein content[30,31]. Therefore, the protein and mRNA expression of MIF in cells was detected. As expected, when P115 was overexpressed or silenced, MIF protein and mRNA in cells were also enhanced or reduced compensatively.

The biological mechanism of MIF on tumor growth includes the induction of growth-related protein expression and inhibition of apoptosis-related protein expression[32]. Jung *et al*[14] demonstrated that MIF interacted with p53 *in vivo* and directly promoted tumorigenesis by inhibiting p53 accumulation. Our data demonstrated that P115 knockdown enhanced the expression of p53, which was considered a result of MIF reduction. It is known that p53 is a classic tumor suppressor gene that can promote cell cycle arrest and apoptosis in response to DNA damage. Absence or down-regulation of p53 can interfere with these important checkpoints for maintaining genetic stability and allows cells to survive and proliferate. This may explain our results where knockdown of P115 led to the inhibition of cell proliferation and apoptosis (results not shown).

To further explore the molecular mechanism of P115 influencing cell growth, key proteins involved in the G_0-G_1 phase relevant signaling pathway were determined. It was reported that the ERK1/2 pathway was necessary for transcriptional induction of cyclin D1 which promoted progression from G_1 to S phase[33]. In addition, Mcm2 and PCNA are both important proteins for initiation of DNA synthesis[34]. As shown in our results, cyclin D1, Mcm2 and PCNA, as well as pERK1/2 were markedly reduced by P115-shRNA, which was consistent with

G_0-G_1 arrest. Researchers have reported that recombinant MIF can activate the ERK-MAP kinase pathway, and subsequently increase cell proliferation rate in fibroblasts and a colon cancer cell line[35]. Therefore, it is concluded that MIF is the key factor in the biological function of P115 in cell proliferation.

In conclusion, our study demonstrates that P115 is overexpressed in gastric cancer tissue and cells. Knockdown of P115 blocks cell proliferation *in vitro*, and the mechanism involves P115 stimulating the secretion of MIF directly by interacting with MIF, subsequently, leading to progression of cell cycle through relevant proteins. Although additional functional studies are required, P115 as well as the interaction between P115 and MIF may be a potential therapeutic target for the treatment of gastric cancer.

COMMENTS

Background

Cancer growth is a highly complex process involving alterations in gene expression and the interaction of many proteins. Golgi-vesicular transport protein P115 is a tether protein that plays an important role in many signal pathways required for cell proliferation. Some studies have reported the function of P115 in intra-Golgi transport, but there are few studies on the role of P115 in cancer cell proliferation and on its biological mechanism.

Research frontiers

The ability of migration inhibitory factor (MIF) to promote tumor progression has been demonstrated. Recently, a yeast two-hybrid interaction was examined to identify the intracellular proteins which might bind to MIF and mediate its secretion, and it was shown that P115 was a binding partner of MIF.

Innovations and breakthroughs

This study showed that over-expression of P115 could significantly enhance the secretion of MIF, which indicated that P115 might be one of the stimuli inducing MIF secretion through a direct interaction. Through interaction with MIF, P115 promoted progression from G_1 to S phase and then influenced the growth of cancer cells. Correspondingly, cyclin D1, Mcm2 and PCNA, as well as pERK1/2 were markedly reduced by P115-shRNA, being consistent with G_0-G_1 arrest.

Applications

The study results suggest that P115 as well as the interaction of P115 and MIF may be a potential therapeutic target for treatment of gastric cancer.

Terminology

Protein-protein interaction: the majority of proteins in living systems function due to interaction with each other in stable or dynamic protein complexes. P115 is a tether protein which has been demonstrated to be involved in intra-Golgi transport. MIF is a secretary protein which plays an important role in regulation of diverse cellular responses.

Peer review

This is a good descriptive study in which authors explore the expression and role of two important proteins in gastric cancer biology. The results are significant and suggest that P115 promote cells proliferation through interaction with MIF and provide a potential therapeutic target for treatment of gastric cancer.

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ORIGINAL ARTICLE

Clinicopathological and biological significance of *cripto* overexpression in human colon cancer

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Abstract

AIM: To assess the clinicopathological and biological significance of *cripto* in human colorectal cancer.

METHODS: Real-time reverse-transcription polymerase chain reaction (PCR) was used to examine *cripto* mRNA levels in primary colon cancer and normal colon tissues as well as normal and metastatic lymph nodes from colon cancers. Human colon cancer LS-174T cells were transfected with *cripto* small interfering RNA (siRNA), and mRNA and protein levels were evaluated using real-time PCR and western blot analysis, respectively. The growth of cancer cells was evaluated using the MTT assay and colony formation in soft agar. Invasion was examined using a Transwell assay, and the expressions of matrix metalloproteinase (MMP)-7 and MMP-9 were determined using western blot assay.

RESULTS: Cripto was significantly overexpressed in primary colon cancer and metastatic lymph nodes. Silencing *cripto* gene expression with *cripto* siRNA resulted in a significant decrease in colony formation in soft agar in the colon cancer cell line LS-174T. Cripto siRNA treatment decreased the migration and invasion capabilities of the colon cancer cell line LS-174T *in vitro*. Furthermore, *cripto* siRNA treatment inhibited the expression of matrix MMP-7 and MMP-9.

CONCLUSION: The results provide evidence that *cripto* siRNA could be an effective approach for the inhibition of cancer cell invasion and migration and thus has potential for use in devising novel preventive and therapeutic strategies for colon cancer metastasis.

Key words: Colon cancer; Invasion; Metastasis; Cripto; Small interfering RNA; Matrix metalloproteinases

Core tip: To assess the clinicopathologic and biological significance of *cripto* as a novel target for colon cancer gene therapy, pathological and *in vitro* studies were carried out. *Cripto* was significantly overexpressed in primary colon cancer and metastatic lymph nodes. *In vitro* studies found that *cripto* siRNA resulted in a significant reduction in colony formation in soft agar and in the migration and invasion abilities of colon cancer cells. Furthermore, *cripto* siRNA led to an inhibition of *MMP-7* and *MMP-9*. These results suggest that the *cripto* gene be useful for devising novel preventive and therapeutic strategies for colon cancer.

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INTRODUCTION

The *cripto* gene is a member of the epidermal growth factor-CFC family of signaling proteins first cloned from the human teratocarcinoma cell line NTERA2[1]. Cripto is overexpressed in most malignant solid tumors, including colon, breast, lung, ovarian, and pancreatic cancers[2-7]. In contrast, crypto is generally absent from or found at low levels in normal tissues. *In vitro*, *cripto* exhibits many of the properties of an oncogene, including transformation of immortalized cells, induction of cell migration, and stimulation of branching morphogenesis[8]. These findings suggest that *cripto* serves an important function in carcinogenesis and in the development of some tumors.

Recent reports show that *cripto* overexpression may be closely related to invasion and metastasis in some human cancers[9,10]. Ertoy *et al*[9] evaluated *cripto* expression in matched sets of non-neoplastic cervical epithelium, primary cervical carcinoma, and metastatic tumors in the lymph nodes using immunopositivity staining. Strong *cripto* immunopositivity was found to be significantly correlated with tumor size and lymphovascular space involvement ($P < 0.05$). More importantly, the level of *cripto* expression increased in metastatic lymph nodes compared with their primary tumors. Despite the clear association between *cripto* overexpression and human breast cancer, the clinicopathological and biological significance of *cripto* overexpression in human colon cancer remains undiscovered.

Colorectal cancer is the third most common malignant neoplasm worldwide[10] and the second leading cause of death attributed to cancer[11]. Despite recent advances in diagnostic and therapeutic measures, the prognosis of colorectal cancer patients with distant metastasis remains poor. Enhanced understanding of the signaling mechanisms that regulate the metastasis of colon cancer may provide important insights with which to establish improved therapeutic strategies.

In this study, we demonstrate that *cripto* is highly overexpressed in primary and metastatic colon cancer tissues. In addition, we demonstrate that RNA interference (RNAi) *cripto* gene expression decreases the proliferation, migration, and invasion capability of colon cancer cell lines *in vitro*. To define the mechanisms underlying *cripto* invasion inhibition, we investigate the effect of *cripto* small interfering RNA (siRNA) transfection on the expression levels of mRNA and proteins of matrix metalloproteinase (MMP)-7 and MMP-9. Based on the results of this study, we conclude that *cripto* is a potential novel target of gene therapy for colon cancer metastasis.

MATERIALS AND METHODS

Tissue specimens and treatment

Thirty-nine paired samples of colon cancer and distant normal colon tissue were obtained from 39 inpatients who had undergone surgery from 2009 to 2010 in the Affiliated People's Hospital of Jiangsu University. Eighteen metastasized lymph nodes were obtained from patients undergoing surgical therapy for the treatment of colon cancer. Tumor histotype and grade of differentiation were defined according to WHO criteria. Clinical and pathological stages were defined according to Dukes Staging' criteria. Inpatients did not receive any chemotherapy or radiotherapy prior to surgery. Eleven normal lymph nodes without evidence of cancer were obtained from patients undergoing carotid endarterectomy. None of these patients had any history or clinical evidence of cancer.

Table 1 Clinicopathologic characteristics of colon cancer patients *n* (%)

Characteristic	No. of patients
Sex	
Male	21 (53.8)
Female	18 (46.2)
Age (yr), mean (range)	62 (35–81)
≤ 65	20 (51.3)
> 65	19 (48.7)
Dukes staging	
A + B	12 (30.8)
C + D	27 (69.2)
Tumor differentiation	
Well	0
Moderate	29 (74.4)
Poor	10 (25.6)

To facilitate real-time reverse-transcription polymerase chain reaction (RT-PCR) analysis, the specimens were identified and bisected. One portion was processed for real-time RT-PCR, whereas the other portion was sent for routine pathology analysis. All specimens were immediately snap-frozen in liquid nitrogen to prevent RNA degradation. The specimens were then stored at -70 ℃ until total RNA processing could be performed. This study was conducted with approval from the Medical Ethical Committee, and all patients provided written informed consent to participate in the study (Table 1).

Cell lines

The human colon cancer cell lines LS-174T and GEO were obtained from the Institute of Cell Biology, Shanghai, China. Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) at 37 ℃ under a 5%CO_2 atmosphere. For 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, cells were plated in 96-well plates at a density of 2×10^3 cells/well. For real-time RT-PCR, cells were seeded in 6-well plates at a density of 1×10^5 cells/well.

RNA isolation and complementary DNA synthesis

Total cellular RNA was isolated from colon cancer cell lines, normal lymph nodes, and lymph nodes from primary colon cancer patients using Trizol. Final RNA pellets were dissolved in 20 μL of diethyl pyrocarbonate-treated water. RNA yield was determined by spectroscopy. For complementary DNA (cDNA) synthesis, 5 μg of total RNA was transcribed with cDNA transcription reagents using 0.5 μg of oligo(dT)18 primer for subsequent quantitative, RT-PCR.

Table 2 Primers of *cripto*, matrix metalloproteinase-7 and matrix metalloproteinase-9

Genes	Forward(5'-3')	Reverse(5'-3')
Cripto	CAATTCGGCCTCGGTCTTC	TTCAGGCAGCAGGTTCTGTTT
MMP-7	AACTCCCGCGTCATAGAAAT	GATACGATCCTGTAGGTGAC
MMP-9	CGGAGTGAGTTGAACCAG	GTCCCAGTGGGGATTTAC

MMP: Matrix metalloproteinase.

Real-time RT-PCR

Real-time RT-PCR analyses were performed on an ABI Prism 7700 sequence detection system (Applied Biosystems, CA, United States). Primers and TaqMan probes were designed using Primer Express™ 1.0 (Applied Biosystems) software, and probes were labeled at the 5' end with the reporter dye molecule FAM (6-carboxy-fluorescein) and at the 3' end with the quencher dye molecule TAMARA (6-carboxytetramethyl-rhodamine). Real-time PCR was conducted in a total volume of 50 μL with 1 × TaqMan Master Mix (Applied Biosystems) and primers. Thermal cycler parameters included one cycle at 95 ℃ for 3 min, 45 cycles involving denaturation at 95 ℃ for 30 s, annealing at 52 ℃ for 45 s, and extension at 72 ℃ for 45 s, followed by a final extension at 72 ℃ for 10 min. The relative amount of cDNA in each sample was calculated by dividing the CT value with the corresponding value of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). All reactions were performed in triplicate. The data were normalized to the internal control gene, GAPDH, to control for RNA preparation. Real time RT-PCR results were analyzed using Q-Gene software[12], which expresses data as mean normalized expression (MNE). MNE is directly proportional to the amount of RNA of the target gene (*cripto* and *MMPs*) relative to the amount of RNA of GAPDH.

Primer design

Primers for *cripto*, *MMP-7*, and *MMP-9* were designed using Perkin-Elmer Primer Express software. The primer sequences are presented in Table 2.

Cripto siRNA sequence

Cripto siRNAs corresponding to *cripto* mRNA with dTdT on 3'-overhangs were designed and chemically synthesized according to the recommendation of the manufacturer (Dharmacon Research, United States). Six SiRNAs targeting the coding sequence of *cripto* mRNA (S1-S6) were used in the current experiment. Information on the *cripto* siRNAs is provided in Table 3. The scrambled siRNA served as a control and its sequences were 5'-UUCUCCGAACGUGUCACGUTdTd-3' and 5'-ACGUGACACGUUCGGAGAATdTdT-3'.

In vitro transfection

Transfection of siRNA was performed using a commercial reagent, oligofecamine (Invitrogen, United States), in 6-well plates following the manufacturer's instructions. Briefly, the day before transfection, confluent layers of cells were trypsinized, counted, and resuspended. Thereafter, 1 × 10^5 of cells were plated into each well of the 6-well plates, such that approximately 70% confluence could be achieved the next day at the time of transfection. Oligofecamine was diluted in serum-free RPMI 1640 and mixed with siRNA at a 1:2 ratio (4 μL of 20 μmol/L of siRNA formulated with 8 μL of oligofecamine). The cells were then incubated for another 48 h. Cell numbers were determined using a hemocytometer before subsequent assays.

MTT assay

Cells plated in 96-well plates were grown in their respective media for 48 h after the addition of siRNA. At each time point, cells were checked visually for growth and proliferation. MTT (Sigma) was then added to the wells, and the cells were incubated at 37 ℃ for 4 h. MTT solubilization solution (10% Triton X-100 in acidic isopropanol, 0.1 mol/L HCl) was added to the wells, and the cells were incubated overnight. Colorimetric measurements were performed using a microplate reader (Molecular Devices) at 560 nm, and the background at 650 nm was subtracted.

Anchorage-independent growth assay

For the anchorage-independent growth experiments, LS-174T cells (1 × 10^4 cells/well) were seeded in 0.3% FBS supplemented with complete culture medium. This suspension was layered over 0.5 mL of 0.8% agar-medium base layer in 24-well plates. After 15 d, the colonies were stained with nitroblue tetrazolium, and colonies larger than 50 μm were acquired using a micro-Scopeman camera system (Moritex Europe Ltd.) and analyzed with Image-Pro Plus (Media Cybernetics) software.

In vitro invasion/migration assay

Transwell migration and invasion assays were performed using LS-174T cells cultured in 12-well plates containing either 8 μm pore Biocoat® control inserts (migration assays) or Matrigel-coated inserts (invasion assays) according to the manufacturer's instructions (Becton Dickinson, Bedford, MA, United States). The membranes were rehydrated with warm serum-free Dulbecco's modified Eagle's medium (1.0 mL/chamber) for 2 h. The upper chamber was filled with 1 × 10^5 cells in L-15 medium containing 5% FBS. The lower chamber was filled with L-15 medium containing 25% FBS as a chemoattractant. After the chambers were incubated for 24 h at 37 ℃ under a 5% CO_2 atmosphere, the non-invading cells were removed from the upper surface of the membrane by scrubbing, and invading cells on the lower surface of the membrane were fixed and stained with hematoxylin and eosin.

The number of cells that penetrated the filter was counted by a technician blinded to the experimental settings in four microscopic fields of each filter under × 20 magnification. The percentage of invasion was expressed as the ratio of the mean cell number from the invasion chamber to the mean cell number from the control chamber according to the manufacturer's recommenda-

Table 3 *Cripto* siRNA sequence

siRNA	Sense (5' → 3')	Antisense (5' → 3')	MW	Position
S1	UUCGGCCUCGGUCUUCCCATT	UGGGAAGACCGAGGCCGAATT	13347.2	175-195
S2	CAGAACCUGCUGCCUGAAUTT	AUUCAGGCAGCAGGUUCUGTT	13317.2	236-256
S3	CUGUGAGCACGAUGUGCGCTT	GCGCACAUCGUGCUCACAGTT	13347.2	314-334
S4	GAGAACUGUGGGUCUGUGCTT	GCACAGACCCACAGUUCUCTT	13332.2	336-356
S5	UGCUGGCACGGUCAGCUCCTT	GGAGCUGACCGUGCCAGCATT	13362.2	396-416
S6	CUACCACCGUCUGCACGUATT	UACGUGCAGACGGUGGUAGTT	13332.2	495-515

tion. The percentage of migration was expressed as the ratio of the mean cell number in control inserts containing S1 siRNA transfected cells to mean cell numbers in control inserts containing untreated cells (untreated cells were given a value of 100%).

Western blot assay for MMP-7 and MMP-9

Seventy-two hours after transfection, cells were washed twice in PBS, and total protein was extracted in 150 mmol/L NaCl, 50 mmol/L Tris·HCl (pH 7.5), 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 5 mmol/L EDTA, 10 mg/mL leupeptin, 1% aprotinin, and 2 mmol/L PMSF. Ten micrograms of protein sample was loaded onto a 10% SDS-PAGE and electroblotted onto a PVDF nylon membrane (Millipore, Bedford). Membranes were blocked in 0.05% Tween 20 (v/v) PBS containing 5% skimmed milk and then incubated with MMP-7, MMP-9, and β-actin antibodies (Santa Cruz Biotechnology). Membranes were then incubated with a HRP-linked goat anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology). Finally, the membrane was reacted with DAB reagent and washed with PBS once protein bands had appeared.

Statistical analysis

Statistical analyses included the independent *t*-test and analysis of variance. Statistical analyses were performed using SPSS 11.5 software (SPSS Inc., Chicago, IL, United States.

RESULTS

Cripto is highly expressed in primary colon cancer

To determine whether or not *cripto* was expressed in human colon cancer, real-time RT-PCR was conducted on 39 paired samples to determine *cripto* mRNA expression levels in clinical tissues. The results showed that *cripto* expression in primary colon cancer samples was significantly higher (mean expression in cancer tissue was more than 16-fold higher; $P < 0.001$) than that in normal tissues.

Cripto is significantly overexpressed in lymph nodes containing metastatic colon cancer

Real-time RT-PCR analysis was performed on 11 normal lymph nodes and 18 lymph nodes containing metastatic colon cancer. The results indicated that *cripto* expression in metastatic lymph nodes was significantly higher (mean expression in cancer tissue was approximately 150-fold higher; $P < 0.001$) than that in normal lymph nodes.

Screening of cripto siRNA

The capability of siRNA to inhibit *cripto* expression was quantified by real-time RT-PCR analysis 48 h following siRNA exposure. S1-S6 (targeting the coding sequence of *cripto* mRNA) showed various suppressant effects on *cripto* mRNA expression in LS-174T cells which express high levels of cripto mRNA[13,14]. Among them, S1, which targets nucleotides 175-195, exhibited the strongest effect. At a concentration of 100 nmol/L, S1 reduced the *cripto* mRNA level by 89% 48 h after the start of transfection (Figure 1). In contrast, the control-scrambled siRNA treatment showed no effect on *cripto* mRNA levels, thus supporting the specificity of *cripto* siRNA. To characterize the potency of S1 further, the dose and time dependency of its effects on *cripto* mRNA in LS-174T cells were examined, and results indicated that S1 downregulated the *cripto* mRNA level in a dose-dependent manner (Figure 2).

Effects of cripto siRNA on proliferation in LS-174T cells in vitro

We then evaluated the biological effects of *cripto* suppression on colon cancer LS-174T cells using different types of assays. The MTT assay showed that cellular proliferation in the monolayer culture was unaffected by either siRNA (Figure 3A). However, colony formation in soft agar was strongly inhibited by treatment with *cripto* siRNA but not by control siRNA (Figure 3B). Figure 3B shows that treatment with *cripto* siRNA induced significant anchorage-independent growth inhibition in a dose-dependent manner (Figure 3).

Downregulation of cripto decreased cancer cell invasion and migration capabilities of colon cancer cell lines in vitro

Colony formation in soft agar is a property closely associated with malignancy. Given the known role of *cripto* siRNA in the downregulation of anchorage-independent growth of LS-174T cells, we attempted to determine whether or not the *cripto* gene contributes to cell invasion and migration in colon cancer. Cell migration and invasion studies were performed using Matrigel matrix assays. Tumor cells require both migration and invasion properties to invade through the Matrigel matrix. Two independent experiments were performed. The results showed that S1 treatment, but not scrambled siRNA treatment,

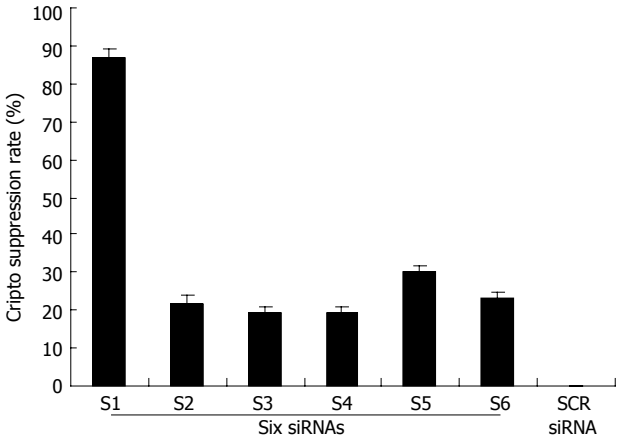

Figure 1 Suppression of *cripto* mRNA by six siRNA transfection in LS-174T cells. The colon cancer cell line LS-174T was transfected with 100 nmol/L of six siRNA after 48-h incubation. Real-time polymerase chain reaction quantification of the relative amount of *cripto* transcript was performed using the *cripto* primers and probe set as described in "Materials and Methods," using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal standard. All data are presented as the mean ± SD of three independent experiments; the level of *cripto* mRNA relative to GAPDH in untreated cells maintained under identical experimental conditions was taken as 100%.

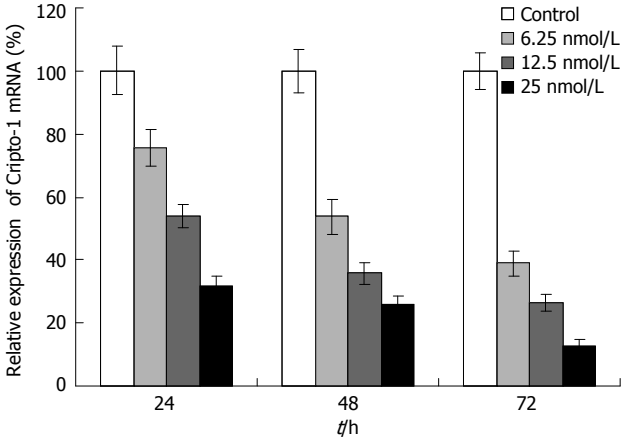

Figure 2 Effects of S1 siRNA on *cripto* mRNA of colon cancer LS-174T cells. The LS-174T colon cancer cell line was transfected with different dose of S1 siRNA for 24, 48, 72 h. *Cripto* mRNA was evaluated by real-time reverse transcription-polymerase chain reaction as described in "Materials and Methods," using GAPDH as internal standard. All data are presented as the mean ± SD of three independent experiments; the level of *cripto* mRNA relative to GAPDH in untreated cells maintained under identical experimental conditions was taken as 100%.

resulted in a significant low level of migration and invasion potential of LS-174T cells (Figure 4).

Effects of cripto siRNA on MMP-7 and MMP-9 expression in colon cancer cells

To explore whether or not the invasiveness of transfected cells was associated with MMP induction, real-time PCR and western blot assays were conducted to detect alterations in the expression level of MMP-7 and MMP-9. As illustrated in Figure 5, *cripto* suppression resulted in decreases in both mRNA and MMP-7 and MMP-9 protein levels compared with those in control cells. Another

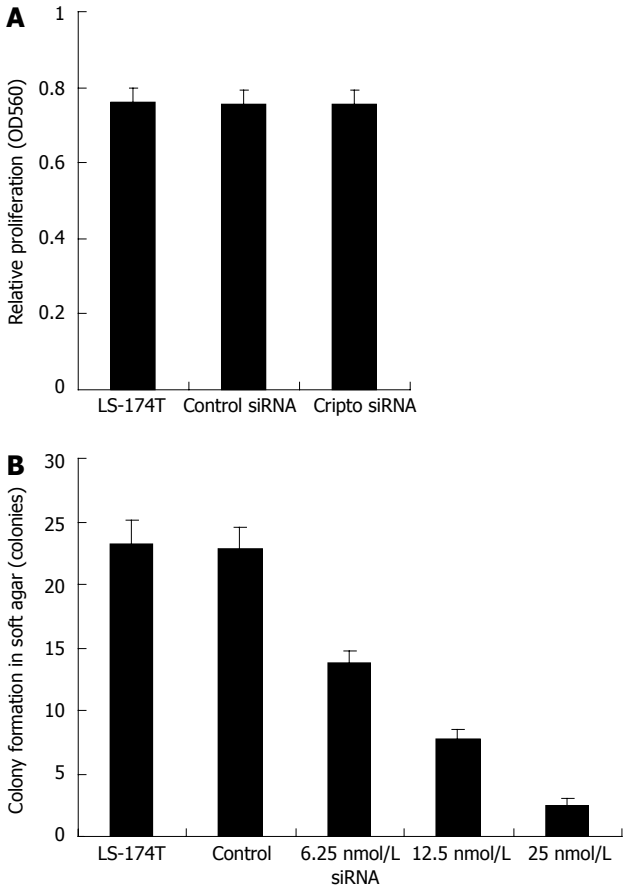

Figure 3 Effects of S1 siRNA on cell proliferation and anchorage-independent growth of colon cancer LS-174T cells. A: S1 siRNA treatment decreases cell proliferation. Cells plated in 96-well plates were grown in their respective media for 48 h after the addition of siRNA. Cell proliferation was examined by MTT assay; B: S1 siRNA treatment reduces anchorage-independent growth of LS-174T cells. The anchorage-independent growth was evaluated by colony formation in soft agar.

experiment on transfection with *cripto* siRNA in the colon cancer cell line GEO also exhibited invasion inhibition and downregulation of *MMP-7* and *MMP-9* expression (data not shown). These results indicate that *cripto* suppression by RNAi could inhibit invasion and migration capabilities by reducing *MMP-7* and *MMP-9* expression in human colon cancer cells.

DISCUSSION

The primary modalities for colon cancer therapy are surgery, radiotherapy, and chemotherapy. One of the main limitations of current treatment modalities is that systemic therapies for metastatic disease are not curative. To improve the choice of therapeutic strategy, the mechanism of invasion and metastasis of colon cancer must be clarified.

The *cripto* gene is known to be overexpressed in numerous solid cancers, and its overexpression appears to be associated with enhanced proliferation and malignant potential[1,2,5]. We found that *cripto* was significantly overexpressed in primary and metastatic colon cancer tissues through real-time RT-PCR. To determine whether or not

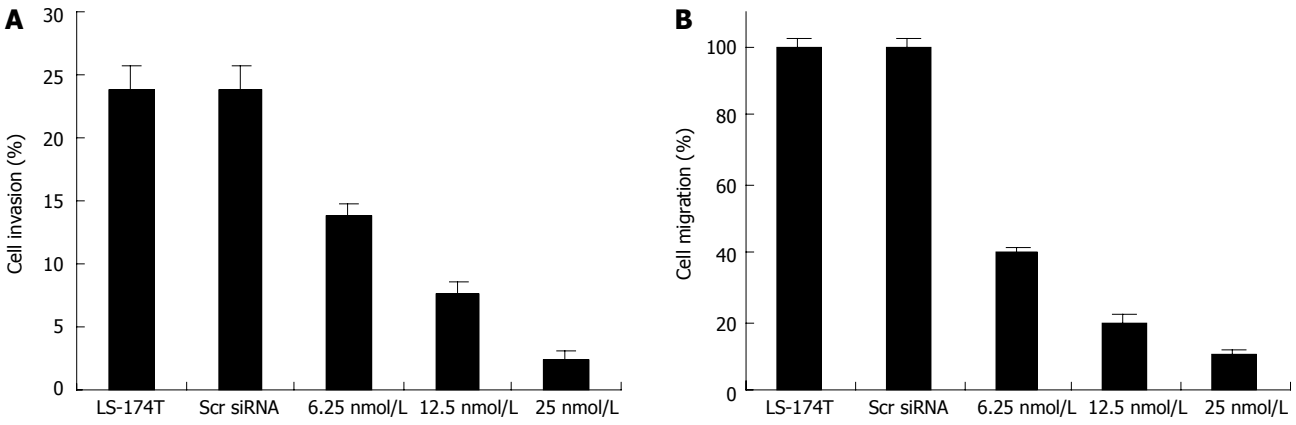

Figure 4 Effects of S1 siRNA on migration and invasion of colon cancer LS-174T cells. The LS-174T colon cancer cell line was used in this experiment. Cell migration was assessed in BioCoat control cell culture chambers. Transwell migration and invasion assays were performed using LS-174T cells cultured in 12-well plates containing either Matrigel-coated inserts (invasion assays) or 8 μm pore Biocoat® control inserts (migration assays), according to the manufacturer's instructions. Control, scrambled siRNA-, and S1 siRNA-treated cells were added to control and Matrigel chambers. A: The percentage of invasion was expressed as the ratio of mean cell number from invasion chamber to mean cell number from control chamber according to the manufacturer's recommendation; B: The percentage of migration was expressed as the ratio of mean cell number in control inserts containing siRNA-treated cells to mean cell number in control inserts containing untreated control cells. Control cells were given a value of 100%.

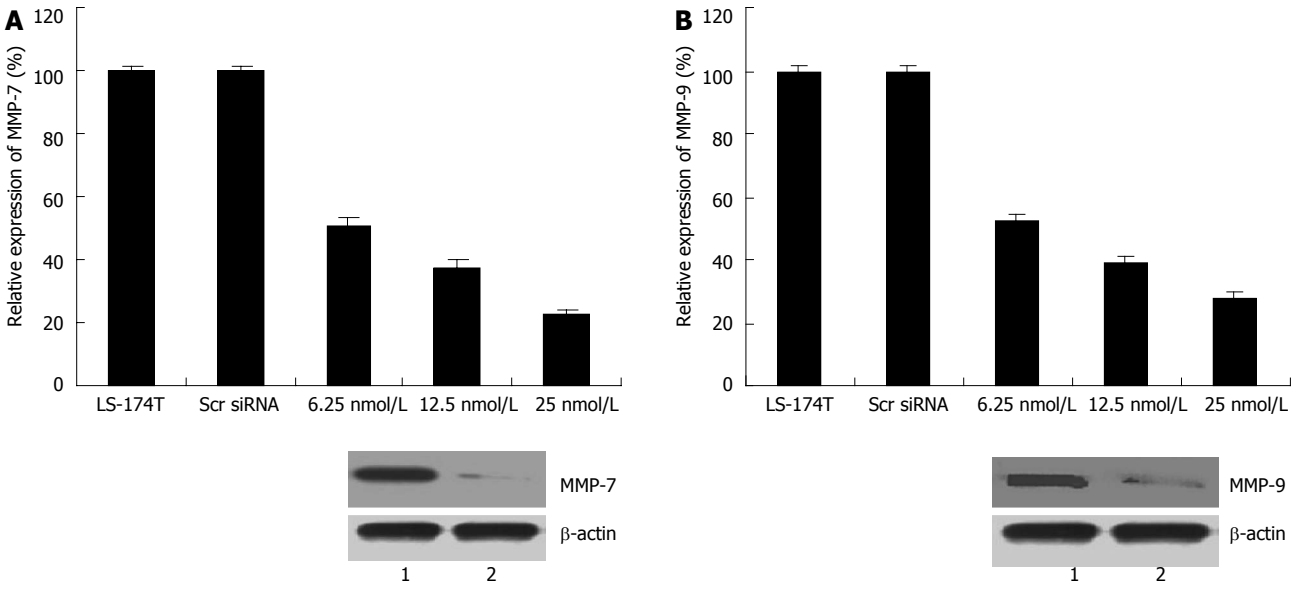

Figure 5 Effects of S1 treatment on matrix metalloproteinase-7 and -9 expression of colon cancer line LS-174T. After cancer cells were treated with S1 siRNA for different times, cells were harvested, and total RNA and proteins were extracted. Real-time reverse transcription-polymerase chain reaction and western blotting were performed to detect mRNA and protein levels, respectively, of matrix metalloproteinase (MMP)-7 and -9. A: Expression level of mRNA and protein of MMP-7 (2 h); B: Expression level of mRNA and protein of MMP-9 (72 h). 1: Scr siRNA; 2: S1(25 nmol/L).

cripto was a potential target for colon cancer gene therapy, the colon cancer cell line LS-174T was treated with *cripto* siRNA, and anchorage-independent growth and the capacity for invasion and migration were determined using different assays. The results showed that cancer cells transfected with *cripto* siRNA inhibited the anchorage-independent growth, invasive capacity, and migration capability of colon cancer cells. Finally, our results provide mechanistic insight into the function of *cripto* in the regulation of invasion and migration through the suppression of *MMP-7* and *MMP-9* expression, which suggests that *cripto* may serve as a novel tumor marker for colon cancer metastasis.

The mechanisms by which *cripto* regulates invasive potential and migration capability remain unclear. Normanno *et al*[15] recently showed that crypto overexpression enhanced invasion capability by inhibiting anoikis in breast cancer. The process of metastasis is complex, occurring in a series of steps, including cell invasion and degradation of the basement membranes and stromal extracellular matrix, ultimately leading to tumor cell invasion and metastasis. MMPs are critically involved in the processes of tumor cell invasion and metastasis[16-19]. The MMPs comprise a family of related enzymes that degrade the extracellular matrix and are considered to be important factors in facilitating tumor invasion. Among

the MMPs, MMP-7 and MMP-9 have been considered important factors in facilitating invasion and metastases in human colon cancer[20-24]. We further investigated whether or not *cripto*-induced invasion of LS-174T cells is mediated through MMP-7 and MMP-9. Real-time RT-PCR and Western blot analysis were performed to detect MMP expression in the colon cancer cell line LS-174T. Cripto siRNA-transfected cells showed significantly low levels of mRNA and MMP-7 and MMP-9 proteins. Data from these results show that the downregulation of *cripto* expression after siRNA treatment decreases MMP expression. To the best of our knowledge, this study is the first to report on the primary mechanism responsible for the decrease in invasion potential observed after *cripto* siRNA treatment.

Based on our studies, we speculate that transfection with *cripto* siRNA decreases invasion and metastasis in human colon cancer through MMP downregulation. Thus, targeting *cripto* for molecular intervention may be an attractive therapeutic strategy for colon cancer. Many studies have proven that RNAi technology siRNA can be used successfully for gene silencing *in vivo*[25,26]. Thus, the application of RNAi mediated by siRNA to knock down *cripto* expression in colon cancer may prove to be a valuable strategy for patients with advanced colon cancer.

COMMENTS

Background

The *cripto* gene is always overexpressed in most malignant solid tumors, including colon, breast, lung, ovarian, and pancreatic cancers. Recent reports showed that *cripto* overexpression may very well be closely related to invasion and metastasis in a few of human cancers, including cervical carcinoma and breast cancer, but the clinicopathologic and biological significance of *cripto* overexpression in human colon cancer remains unclear.

Research frontiers

To explore the clinicopathologic and biological significance of *cripto* as a novel target for colon cancer gene therapy, pathological and *in vitro* studies were carried out using RNA interference (RNAi). The results suggest that the *cripto* gene may be useful for devising novel preventive and therapeutic strategies for colon cancer.

Innovations and breakthroughs

The results showed that RNAi *cripto* can decrease cell migration and invasion by inhibiting matrix metalloproteinases.

Applications

The results here suggested that the detection of *cripto* is helpful in understanding the development of colorectal carcinoma, and that *cripto* siRNA could be an effective approach for the inhibition of invasion and migration of human colon cancer.

Terminology

RNAi's main function is to adjust and shut down gene expression and regulate all kinds of activities of cells.Small interfering RNA (siRNA) is generated within the cell in the process of RNAi, which consists of fragments of about 21-25 nuclear acids of the double-stranded RNA molecule.

Peer review

The biological significance of *cripto* in the occurrence and development of colorectal carcinoma *in vivo*, *in vitro* were investigated. The results showed that *cripto* was significantly overexpressed in primary colon cancer and metastatic lymph nodes. Furthermore, the molecular mechanism of invasion and the regulation of *cripto* gene expression in colon cancer cells were explored by RNA interference technology. The study found, *MMP-7* and *-9* gene is involved in the regulation of *cripto* invasion of colorectal cancer cells. There is important innovation and scientific significance of the study.

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BRIEF ARTICLE

Randomized trial in malignant biliary obstruction: Plastic *vs* partially covered metal stents

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Abstract

AIM: To compare efficacy and complications of partially covered self-expandable metal stent (pcSEMS) to plastic stent (PS) in patients treated for malignant, infrahilar biliary obstruction.

METHODS: Multicenter prospective randomized clinical trial with treatment allocation to a pcWallstent® (SEMS) or a 10 French PS. Palliative patients aged ⩾ 18, for infrahilar malignant biliary obstruction and a Karnofsky performance scale index > 60% from 6 participating North American university centers. Primary endpoint was time to stent failure, with secondary outcomes of death, adverse events, Karnofsky performance score and short-form-36 scale administered on a three-monthly basis for up to 2 years. Survival analyses were performed for stent failure and death, with Cox proportional hazards regression models to determine significant predictive characteristics.

RESULTS: Eighty-five patients were accrued over 37 mo, 42 were randomized to the SEMS group and 83 patients were available for analyses. Time to stent failure was 385.3 ± 52.5 d in the SEMS and 153.3 ± 19.8 d in the PS group, $P = 0.006$. Time to death did not differ between groups (192.3 ± 23.4 d for SEMS *vs* 211.5 ± 28.0 d for PS, $P = 0.70$). The only significant predictor was treatment allocation, relating to the time to stent failure ($P = 0.01$). Amongst other measured outcomes, only cholangitis differed, being more common in the PS group (4.9% *vs* 24.5%, $P = 0.029$). The small number of patients in follow-up limits longitudinal assessments of performance and quality of life. From an initially planned 120 patients, only 85 patients were recruited.

CONCLUSION: Partially covered SEMS result in a longer duration till stent failure without increased complication rates, yet without accompanying measurable benefits in survival, performance, or quality of life.

Key words: Randomized; Biliary; Obstruction; Stent; Plastic; Metal; Palliative; Common bile duct

Core tip: This randomized trial is one of very few comparing partially covered self-expandable metal stent (SEMS) to 10 French plastic stent (PS) in the contemporary palliation of malignant biliary obstruction. In 85 patients, time to stent failure was significantly longer (385.3 ± 52.5 d) in SEMS *vs* PS (153.3 ± 19.8 d), P = 0.006. Time to death did not differ (192.3 ± 23.4 d for SEMS *vs* 211.5 ± 28.0 d for PS, P = 0.70). Amongst other measured outcomes, only cholangitis differed and was more common in PS (4.9% *vs* 24.5%, P = 0.029).

Moses PL, AlNaamani KM, Barkun AN, Gordon SR, Mitty RD, Branch MS, Kowalski TE, Martel M, Adam V. Randomized trial in malignant biliary obstruction: Plastic *vs* partially covered metal stents. *World J Gastroenterol* 2013; 19(46): 8638-8646 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i46/8638.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i46.8638

INTRODUCTION

Malignant obstructive jaundice is associated with many symptoms that negatively impact quality of life such as anorexia, pruritus and malabsorption[1-3]. Endoscopic retrograde cholangio-pancreaticography (ERCP) with placement of a biliary stent is the procedure of choice for palliation of infrahilar common bile duct (CBD) malignant biliary obstruction[4].

Both plastic and self-expandable metallic stents can palliate malignant biliary obstruction, and although randomized trial data have shown uncovered metallic stents to remain patent for longer periods compared to plastic stents[3], the latter remain widely utilized[3,5,6] at least in part due to their lower upfront costs. The more recently introduced covered self-expandable metallic stents remain poorly studied in a randomized clinical trial setting, and may be associated with added complications such as pancreatitis and cholecystitis, as well as stent migration. This holds true for both fully covered and partially covered stents[7-15].

The primary aim of our study was thus to compare the stent patency's of a partially covered metal stent and a commonly used plastic stent in a randomized controlled trial for patients with low to mid-CBD malignant biliary obstruction. We additionally sought to better characterize the safety of the partially covered metal stent and attempted to identify clinical variables that would allow clinicians to choose a metallic or plastic biliary stent.

MATERIALS AND METHODS

Study design and randomization

The study was a randomized clinical trial. Randomization was performed using sealed envelopes in which patients were allocated in a 1:1 proportion to either a partially covered Wallstent® Endoscopic Biliary Endoprosthesis with Permalume™ covering comprised of two components: the implantable metallic stent and the Unistep™ Plus Delivery System, (Boston Scientific, Natick, MA, United States) (self-expandable metal stent, SEMS), or a 10 French (Fr) Amsterdam-type polyethylene plastic stent (PS) biliary stent. The sealed envelopes were opened only at the time of intent of stent insertion in the ERCP suite after confirmation that all selection criteria had been fulfilled. The allocation sequence was performed centrally and patient enrolment and participant assignment was carried out by a third party not directly involved with the patient's care or the measurement of outcomes. Neither patient, treating team, or the evaluators of outcomes were blinded to treatment allocation due to the nature of the intervention and follow-up care required. Each investigator received written approval for the study from his respective Institutional Review Boards prior to study initiation and patient enrollment. The trial did not require prior registration as it was started before 2004 and both stents are FDA approved.

Study population

Inclusion criteria were age 18 or older, and the provision of a signed written voluntary informed consent form approved by the Institutional Review Boards at participating centers. All patients demonstrated laboratory, imaging and/or histological evidence of malignant biliary obstruction. The cause of obstruction could be any intrinsic or extrinsic malignancy extending no more proximal than 1cm below the common hepatic ductal bifurcation. A Karnofsky performance scale was applied. A Karnofsky score > 60% is a validated measure of patient function, previously used in Pancreatico-biliary cancer patients[16]. Patients were required to haven anticipated life expectancy that would allow for completion of full follow-up. Exclusion criteria were jaundice related to intrahepatic cholestasis or obstruction, or a prior attempt at a curative surgical resection for the biliary obstructing lesion. There were 6 participating North American university centers (Fletcher Allen Health Care at the University of Vermont, McGill University Health Centre, St. Elizabeth's Medical Center, Dartmouth Hitchcock Medical Center, Duke University Medical Center, and Thomas Jefferson University).

Outcome measures

The primary endpoint of the trial was the time to occurrence of stent failure as defined by the appearance of one or more cholestatic symptoms accompanied by a 50% increase in bilirubin from the lowest post-stent insertion value recorded prior to this follow-up event, and/or cholangitis (defined as the new onset of pain, fever, and jaundice) whether associated with a stent replacement or not. Repeat ERCP for stent replacement or suspected obstruction using a stent of any type was con-

sidered to represent a stent failure. Secondary outcomes were death, cholestatic symptoms, laboratory data, technical success (defined as the successful delivery and deployment of the initial stent to the desired location in the biliary tree) measured at the time of the procedure, the presence of adverse events, and the Karnofsky performance score. We also administered the short form (SF)-36 general quality of life measurement scale, which is a questionnaire measuring patient's perceptions about functional health and well-being previously administered to bilio-pancreatic cancer patients[1].

Interventions and follow-up management

ERCP was performed by experienced endoscopists; stents were placed with or without prior dilatation or sphincterotomy after sealed-envelope randomization to stent type was done after confirmation of obstruction meeting inclusion criteria. The patient then received either the SEMS or the PS. The length of each type of stent was determined by the biliary anatomy and left to the discretion of the endoscopist as part of the medical effectiveness philosophy of the trial thereby enhancing generalizability of the results. A cholangiogram was performed to document stent patency and position. No prophylactic antibiotics were used. Each patient had one- and three-month follow-up, followed by quarterly scheduled follow-up sessions up to 2 years following stent insertion.

Failed plastic stents were replaced with covered metal stents, while failed covered metal stents were replaced with either one or more plastic or metal stent(s) inserted through the metal stent. The decision for the choice of stent type following stent failure was left to the discretion of the endoscopist and recorded.

Data collection

Dedicated, standardized electronic case report forms were completed by trained research assistants and downloaded into a web-based remote data entry repository. Internal validity of recorded data and missing data quality checks were performed centrally by trained research personnel. At baseline, investigation variables included any significant medical history, the tumor type, stage, and location, the date of diagnosis, the length and maximum diameter of stricture, and administration of any prior anticancer treatment, as well as the Karnofsky Score. Variables assessed at baseline and at periodic follow-up visits (months 1, 3, 6, 9, 12, and if the patient survived, 3-monthly up to month 24) following the index procedure included a cholestatic symptom assessment, the use of any adjuvant treatment such as radiation and/or chemotherapy, laboratory test results (chemistry, hematology), and the Karnofsky index. All adverse events were recorded, including the occurrences of cholangitis, pancreatitis, and cholecystitis using standardized definitions[17].

Sample size calculation

The planned enrollment was 120 patients. Sample size predictions were calculated using a model of binomial proportions and independent samples. Assuming a 25% improvement in stent patency duration with expandable metallic stenting and using a 1-sided type I error rate of 5% and a type II error rate of 20%, approximately 60 patients were thought to be needed in each group.

Statistical analysis

Amongst descriptive variables, continuous variables are reported as means and standard deviations as well as medians where appropriate, and categorical variables as proportions. Inferential testing was carried out using *t*-tests for continuous and χ^2 for categorical variables. Karnofsky scores and quality of life scores were assessed for both intra- and between-group differences comparing baseline values to the last visit on record at the 1-mo, the 3-mo visit, and the 6-mo visits; both within and across groups. We used a *t*-test with either the pooled or Satterthwaite method, depending on the results of the equality of variances test at each follow-up period.

Survival analyses were performed for both stent failure and patient survival using both intention-to-treat (ITT) and per protocol (PP) analyses. In the first group, only subjects who had at least a 50% drop in bilirubin at 1 mo were included. In the second, all subjects were included as originally randomized. Kaplan-Meier curves were created for the SEMS and PS groups, and compared with a log-rank test. Cox proportional hazards regression models were also used to determine if significant covariates were associated with either time-to-stent failure or time-to-death. The proportional hazards assumption was tested with the use of a Kolmogorov-Smirnov Supremum test. The following covariates were included for these analyses in addition to stent randomization group: obstruction (for prediction of mortality), tumor type, known metastatic cancer, chemotherapy or radiation therapy, and the baseline Karnofsky score. Covariates that were associated with the outcome with a *P*-value of 0.15 or less in a univariate model were entered into a multivariable model. There was no planned interim analysis.

RESULTS

Patient population

A total of 85 patients were accrued over 37 mo. The study was closed prior to completion of enrollment of the estimated 120 patients due to a marked slowing of patient accrual (trial fatigue). Of the 85 patients included, 42 were randomized to the SEMS group, and 43 to the PS group. Three patients had evaluable baseline patient data but were excluded from further analyses because of inclusion protocol violations (2 were never stented, and one received a metal covered stent when in fact randomized to plastic stent). Of the 82 patients with analyzable outcomes data, 41 received a SEMS and 41 a PS; the CONSORT diagram is shown in Figure 1. Population characteristics at baseline for both groups are shown in Table 1.

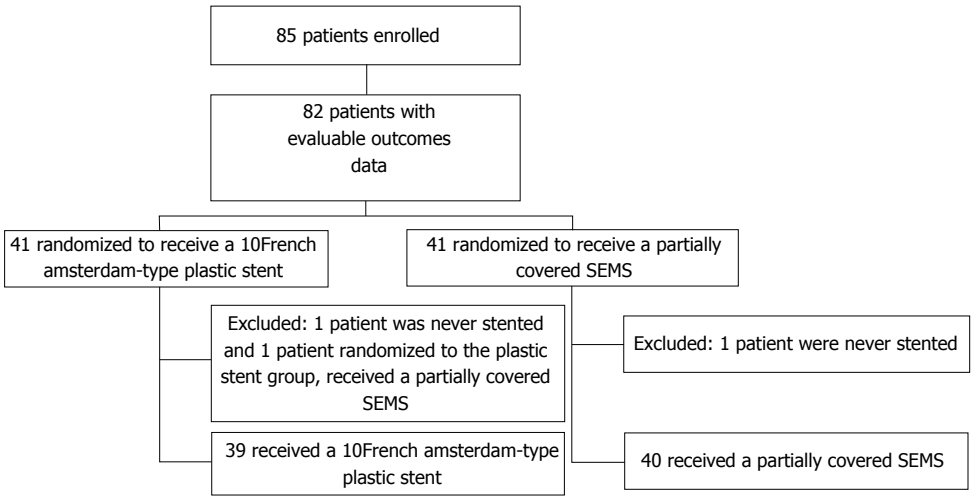

Figure 1 Consolidated standards of reporting trials diagram. SEMS: Self-expandable metal stent.

Twenty-nine point eight percent were inpatients; amongst these, the mean hospital stays related to the procedure were 2.5 ± 1.6 d in the SEMS group, and 4.9 ± 4.7 d in the PS group.

The mean length of Wallstents used was 61.4 ± 11.2 mm (median: 60 mm, range: 40-80 mm); 95.1% patients received a 10Fr diameter and 4.9% an 8F diameter. In the PC group, the stent length was 76.0 ± 18.2 mm (median: 70, range: 50-120 mm); all patients had a 10Fr diameter.

Primary outcome results

In ITT analysis, the time to stent failure was 385.3 ± 52.5 d in the SEMS and 153.3 ± 19.8 d in the PS group (P = 0.006) (Figure 2A). Corresponding results were 396.5 ± 56.8 d and 164.3 ± 24.1 d, respectively (P = 0.025) using a PP approach. After adjustment for possible confounding variables, in ITT analysis, the only independent significant predictor of a failed stent was the stent group allocation (HR = 0.29, 95%CI: 0.12-0.75, P = 0.011); similar findings were noted with the PP analysis (HR = 0.22, 95%CI: 0.06-0.80, P = 0.013)

Secondary outcome

Procedural outcomes: No differences in intra-procedural events were noted. Overall, 69.4% of all stent insertions were carried out in an out-patient setting. Optimal stent insertion and positioning was noted in 95.3% of patient with in the SEMS and 97.4% of patients in the PS groups, respectively. The length of the SEMS used was 61.4 ± 11.2 mm (median: 60 mm, range: 40-80 mm) and a diameter of 59.64 ± 11.2 mm (median: 60 mm, range: 40-80 mm); 95.1% patients received a 10Fr diameter and 4.9% a 8F diameter. In the PC group, the stent length was 76.0 ± 18.2 mm (median: 70 mm, range: 50-120 mm); All patients had a 10Fr diameter. Sphincterotomy was carried out prior to stent insertion in 18.8% of cases, and balloon dilatation in 3.5%. The distal end of the stent was positioned outside the CBD into the duodenum in 93.0%. Some form of tissue sampling was carried out at the time of ERCP in 56.0% of patients.

Time to death: The time to death did not differ between both groups: 192.3 ± 23.4 d for SEMS *vs* 211.5 ± 28.0 d for PS (P = 0.70) using an ITT approach (Figure 2B). Similar conclusions were reached using the PP approach 248.5 ± 26.8 d *vs* 251.3 ± 32.5 d, respectively (P = 0.66). After adjustment for possible confounding variables, no significant predictor of time to death was found in ITT or PP analysis.

Additional secondary outcomes: Complications including the development of pancreatitis, cholangitis, and cholecystitis are (2.4% *vs* 2.4%, P = 1.0000; 4.9% *vs* 24.5%, P = 0.029; 4.8% *vs* 0.0%, P = 0.4741). Only cholangitis differed, with a greater frequency in the PS group.

The percentage reduction in bilirubin value from baseline to the 1-mo visit was no different in SEMS than in the PS group [74.0%, (95%CI: 60.0-87.9) *vs* 63.7% (95%CI: 45.5-81.9), respectively, P = 0.37]. No statistical differences in Karnoksy performance scores were noted between the two treatment groups when comparing the differences in scores for the last, 1, 3, and 6 mo visits compared to baseline. In the pre-planned paired analysis to assess intra-group differences, patients receiving the SEMS, showed significant improvements noted at 6 mo and at the last available visit (P = 0.015, and P = 0.022, respectively). There were also significant improvements noted for patients in the PS group compared to baseline both at 1 mo and at the last available date of follow-up (P = 0.045, and P = 0.0014, respectively; full data available upon request).

Overall, 29.4% of patients had one or more cholestatic symptoms at follow-up, 24.3% for the SEMS group and 33.3% for the PS group (P = 0.213). Additional symptom reporting showed no difference between both groups with regards to individual cholestatic symptoms (data not shown).

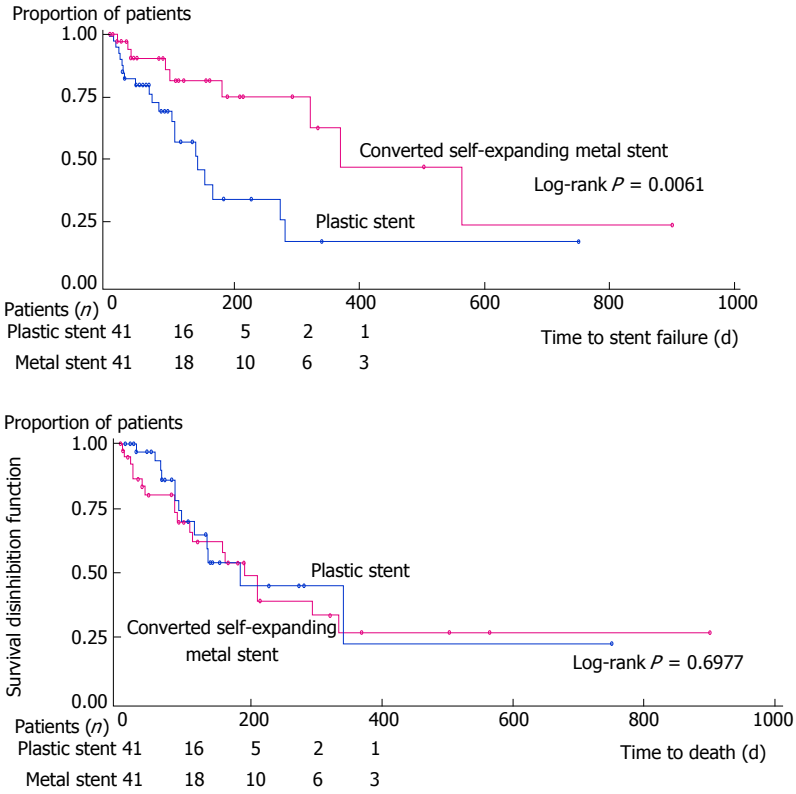

Figure 2 Survival analysis. A: Time to stent failure for partially covered self-expanding metal stent and plastic stent patient groups; B: Time to death for partially covered self-expanding metal stent and plastic stent patient groups.

Quality of life-SF-36 measures: Seventy-four patients answered the quality of life questionnaires over a total of 174 visits during a 12-mo follow-up (31 patients answered only once to the SF-36 questionnaire, 17 answered to two questionnaire and 13 responded to 3 questionnaires). Among these, 38 had received a SEMS and 36 a PS. At baseline, patients in the SEMS group exhibited lower means than those in the PS group for all 8 summary scores, indicating worse quality of life parameters; the differences however were not statistically significant except for physical functioning (46.4 *vs* 63.9, $P = 0.008$). The SEMS group scores improved gradually such that, by 9 mo, most were arithmetically greater than scores from the PS group although without significant differences. There remained, however, only a very small number of patients able to complete the questionnaires in follow-up (9 patients at 9 mo, and 5 at 12 mo). In paired analysis, statistical significant improvements were noted amongst SEMS patients in physical functioning (6 mo *vs* baseline), and vitality (1 mo *vs* baseline). Significant bettering of quality of life was noted amongst PS patients at 1 month *vs* baseline for bodily pain, social functioning, and mental health, as well as in vitality for the 9 mo *vs* baseline comparison (full quality of life scores are available upon request).

DISCUSSION

Stenting for malignant biliary obstruction remains principally a palliative procedure[6,7]; temporary stenting until the time of exploratory or potentially curative surgery is performed (with the advent of useful adjuvant treatment methods), although the efficacy of this approach remains unproven and may in fact be harmful[18-20]. RCT data have suggested the superiority of uncovered metal over plastic biliary stenting[3] owing to the larger internal luminal diameter, thus preventing premature blockage from bacterial biofilm encrustation and sludge formation[21]. Indeed, a Cochrane meta-analysis of 5 trials by Moss *et al*[7] concluded that uncovered metal stents had a lower risk of recurrent biliary obstruction than plastic stents (RR = 0.52, 95%CI: 0.39-0.69), with no difference in technical or therapeutic success, complications or 30-d mortality. An additional trial performed since, also confirmed the superiority of uncovered SEMS over Tannenbaum plastic stents[22], a plastic stent variant without side holes that may contribute to prolonged plastic stent patency.

Although these trials assessed uncovered metal biliary stents, these conclusions were largely presumed to be generalizable to (partially and completely) covered metal stents, and probably is the reason for a paucity of studies examining this latter comparison. This assumption, however, can be questioned and is of contemporary significance for two reasons: (1) plastic biliary stents remain very commonly inserted as initial method of stenting in the face of increasing use of covered and uncovered metal stents for non hilar biliary obstruction[6]; and (2)

Table 1 Patient characteristics at baseline

Characteristic		Partially covered SEMS (n = 42)	10-French polyethylene plastic stent (n = 43)	P value
Gender (male)		51.2%	50.0%	0.9119
Age (yr)		70.8 ± 12.9	73.3 ± 10.7	0.3896
Co-morbidities:	Cardiovascular	53.7%	47.5%	0.5676
	Respiratory	22.0%	20.0%	0.8209
	Neurologic	19.5%	22.5%	0.7343
	GI, liver, biliary	75.6%	77.5%	0.8362
	Renal, urinary	15.0%	30.0%	0.0982
	Musculoskeletal	25.0%	35.0%	0.3148
	Endocrine	47.5%	30.0%	0.0976
Cholestatic symptoms		97.5%	100.0%	0.2968
	Jaundice	85.4%	97.5%	0.0453
	Clay-colored stools	36.6%	52.5%	0.1404
Abdominal pain	Abdominal pain	53.7%	40.0%	0.2056
Pruritus		51.2%	50.0%	0.9119
Dark urine		75.6%	75.0%	0.9489
Fever	Fever	9.8%	5.0%	0.3972
Constitutional symptoms				
	Weight Loss	73.2%	47.5%	0.0155
	Anorexia	51.2%	50.0%	0.9119
Obstruction location	Papilla	2.78%	7.9%	0.2951
	Distal common bile duct	72.2%	47.4%	0.0198
	Mid common bile duct	22.2%	39.5%	0.0845
	Proximal common bile duct	2.8%	5.3%	0.5595
Type of primary tumor	Ampullary carcinoma	2.6%	7.5%	0.3036
	Cholangiocarcinoma	0.0%	5.0%	0.1422
	Gallbladder adenocarcinoma	2.6%	2.5%	0.9767
	Metastatic Cancer	10.3%	7.5%	0.6501
	Pancreatic adenocarcinoma	69.2%	67.5%	0.8662
	Other	0.0%	2.5%	0.3024
	Unknown	15.4%	7.5%	0.2519
Metastat. cancer prim. location	Colon	28.6%	0.0%	0.0002
	Lung	28.6%	20.0%	0.355
	Other	42.9%	80.0%	0.0004
Tumor stage	T1	4.0%	19.2%	0.0292
	T2	32.0%	11.5%	0.0217
	T3	16.0%	42.3%	0.0077
	T4	48.0%	26.9%	0.0443
Nodes	N0	36.4%	61.9%	0.0187
	N1	63.6%	38.1%	0.0187
Metastatic tumor	M0	29.2%	36.0%	0.5038
	M1	70.8%	64.0%	0.5038
Chemotherapy or radiation		11.1%	8.82%	0.7255
Laboratory data	Alkaline phosphatase (IU/L)	630.5 ± 347.7	532.7 ± 331.4	0.1486
	Bilirubin (mg/dL)	9.56 ± 6.99	11.33 ± 7.82	0.3082
	Hematocrit	43.97% ± 50.36%	37.06% ± 5.95%	0.3280
	Hemoglobin (g/dL)	12.01 ± 1.63	12.39 ± 2.09	0.3305
	INR	1.17 ± 0.19	1.25 ± 0.36	0.5922
	AST (IU/L)	168.64 ± 98.34	191.26 ± 149.25	0.6687
	ALT (IU/L)	240.03 ± 178.35	265.03 ± 240.48	0.8926
Karnosky performance scores		81.8 ± 10.8	82.0 ± 12.03	0.9151

SEMS: Self-expandable metal stent; INR: International normalized ratio; AST: Aspartate aminotransferase; ALT: Alkaline phosphatase.

covered metal stents are reported to exhibit greater rates of migration, and perhaps other complications (such as cholecystitis and pancreatitis) compared to uncovered metal stents[5,11,23]. Both these realizations justify the aims of the current trial.

Only two randomized controlled trials have compared plastic to covered metal stents. In the multicenter trial by Isayama *et al*[24], investigators compared a covered metal biliary stent to a rarely used type of plastic stent with a double lumen (found to be superior to polyethylene stents with regards to stent patency[25]) in patients with lower biliary malignant obstruction attributable to pancreatic head cancer. In the Isayama multicentric trial[24], the cumulative stent patency was significantly greater in the covered metal stent group: the respective mean and median stent patency durations were 285 and 419 d, *vs* 202 and 133 d observed for the plastic stent group patients respectively (P = 0.0072). Interestingly, the covered metal stent group experienced more frequent cholecystitis (4 *vs* 0), pancreatitis (1 *vs* 0), and migration (5 *vs* 1), although these differences did not, at least taken separately, achieve statistical significance.

These results validate the findings of the current trial that noted, using life-table analysis, that the time to stent failure was 385.3 ± 52.5 d in the SEMS, and 153.3 ± 19.8 d in the PS group (P = 0.006). Times to stent occlusion were all shorter, although the between-group differences remained, in the only other randomized trial assessing plastic *vs* covered metal stents by Soderlund *et al*[26]. In that study, 22 of 51 plastic stent and 9 of 49 covered metal stent group patients (P = 0.009) developed stent failure after medians of 1.1 and 3.5 mo (P = 0.007), with median patency times of 1.8 mo *vs* 3.6 mo (P = 0.002), respectively.

Even though insertion of a plastic stent is favored in patients with an estimated short survival, such as those with large tumors (over 30 mm), liver metastases, younger age, or adenocarcinoma histology[27-29], summary RCT data have shown that infrahilar biliary stenting, either pre-operatively or as sole palliation, does not improve mortality[3,18,19]. Interestingly, however, an observational trial and an as yet unpublished additional meta-analysis have suggested improvement in survival using expandable metal stent technologies, yet remain unconfirmed[30,31]. Furthermore, while other such comparisons have failed to demonstrate such a benefit[5], two trials have recently suggested a patient survival benefit with the percutaneous insertion of covered rather than uncovered metal stents for infrahilar biliary obstruction, due to pancreatic cancers[15] and cholangiocarcinomas[32].

Despite difficulty in accrual leading to early termination of the study including 85 patients and not the projected 130 patients, the strengths of the current trial include the multi-institutional participation, the medical effectiveness design, and the adopted ITT analytical approach that all increase the generalizability of results. The *a priori* standardized definitions and independent measurements of outcomes all strengthen the validity, minimizing the chance of bias. Life table analysis and multivariable adjustment further ensure the clinical relevance of the findings. Other than improved stent patency, only the outcome of cholangitis differed amongst both groups, occurring more frequently in the plastic stent group. Cholecystitis was a rare outcome, as was pancreatitis, and the trial was not powered to demonstrate any differences in these less frequent endpoints. No differences in procedural outcomes were noted. The between-group quality of life comparisons were limited by the small number of survivors, yet the pre-post stenting analyses within each group using paired analyses confirm what few trials have shown: that a number of quality of life domains improve following successful biliary drainage[1,33,34], and that such benefits were observed both with metal and plastic stents. Post-stent insertion improvements in functional status using Karnovsky scores were also noted at 1 mo and extended to the last available patient visit recorded.

These results, taken as a whole, suggest that the observed benefits of the SEMS studied over the common type of PS used as comparator are attributable to the prolonged stent patency. The resulting decreased rates of cholangitis outweigh any possible risks attributable to increased stent migration, pancreatitis or cholecystitis. Further characterization of optimal patient groups may relate to issues such as cystic duct involvement that predicts cholecystitis[23,35].

Perhaps just as relevant as efficacy findings are cost-effectiveness issues that are being analyzed separately as part of the current trial. Indeed, past cost-effectiveness modeling have suggested that the presence of distant metastases, especially if numerous, is associated with shorter survival time in patients with pancreatic cancer, and that a metallic stent should not be used in this type of patients[27,36,37]. Rather, SEMS should be reserved for patients expected to live for at least 6 mo[38,39]. Nonetheless, as mentioned earlier, plastic stents remain commonly inserted, at least in part due to their lower upfront costs compared to their metal expandable equivalents.

In conclusion, the present study confirms that insertion of a partially covered SEMS for patients with infrahilar biliary obstructing tumors results in a longer duration until stent failure as compared to a commonly used plastic stent (in this case, an Amsterdam-type polyethylene stent) without increased complication rates. There were no measurable benefits in survival, performance, or quality of life. Additional trials and meta-analytical evaluation are required to more confidently assess these important additional patient outcomes.

COMMENTS

Background

Despite existing evidence in the literature favoring metal over platis stenting in distal biliary obstruction palliation, few data exist assessing partially covered metal stents, especially in a contemporary setting.

Research frontiers

Plastuc stent palliation remains widespread, while partially covered metal biliary stents appear to migrate more frequently than their uncovered counterparts, but have gained popularity.

Innovations and breakthroughs

There have been 2 previously published studies in the literature that have compared partially covered metal to plastic biliary stenting with limited generalizability, suggesting the superiority of the metal stent alternative.

Applications

The current article validates the conclusion that partially covered metal stents result in a significantly longer time to stent patency, without a prolonged survival time in patients undergoing palliation for distal biliary malignant obstruction. Additional and summary data are required to confirm the robustness of the latter finding, while the advent of completely covered metal stents require further assessments, owing to the theoretical benefit of decreased stent in growth versus the possible increased risk of stent migration.

Peer review

The findings of this randomized trial appear to confirm that of two previous studies in the literature and is limited by the small sample size, even though a signifcant difference in the primary outcome measure was noted. The achieved statistical power limits the interpretation of other endpoints.

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BRIEF ARTICLE

Clostridium difficile-associated disease: Adherence with current guidelines at a tertiary medical center

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Abstract

AIM: To assess adherence with the the Society for Healthcare Epidemiology of America (SHEA)/ the Infectious Diseases Society of America (IDSA) guidelines for management of *Clostridium difficile* (*C. difficile*)-associated disease (CDAD) at a tertiary medical center.

METHODS: All positive *C. difficile* stool toxin assays in adults between May 2010 and May 2011 at the University of Maryland Medical Center were identified. CDAD episodes were classified as guideline adherent or non-adherent and these two groups were compared to determine demographic and clinical factors predictive of adherence. Logistic regression analysis was performed to assess the effect of multiple predictors on guideline adherence.

RESULTS: 320 positive *C. difficile* stool tests were identified in 290 patients. Stratified by disease severity criteria set forth by the SHEA/IDSA guidelines, 42.2% of cases were mild-moderate, 48.1% severe, and 9.7% severe-complicated. Full adherence with the guidelines was observed in only 43.4% of cases. Adherence was 65.9% for mild-moderate CDAD, which was significantly better than in severe cases (25.3%) or severe-complicated cases (35.5%) ($P < 0.001$). There was no difference in demographics, hospitalization, ICU exposure, recurrence or 30-d mortality between adherent and non-adherent groups. A multivariate model revealed significantly decreased adherence for severe or severe-complicated episodes (OR = 0.18, 95%CI: 0.11-0.30) and recurrent episodes (OR = 0.46, 95%CI: 0.23-0.95).

CONCLUSION: Overall adherence with the SHEA/IDSA guidelines for management of CDAD at a tertiary medical center was poor; this was most pronounced in severe, severe-complicated and recurrent cases. Educational interventions aimed at improving guideline adherence are warranted.

Key words: *Clostridium difficile*; Metronidazole; Vancomycin; Adherence to the Infectious Diseases Society of America Guidelines; Hospital Acquired Infections

Core tip: This study assesses a tertiary care medical center's adherence with updated guidelines on the management of *Clostridium difficile* (*C. difficile*)-associated diseases in adults. We found that overall adherence is poor, especially in patients with severe disease. Factors associated with poor adherence and limitations of current guidelines are identified. Our data suggests that educational interventions aimed at improving *C. difficile* guideline adherence are warranted.

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INTRODUCTION

Clostridium difficile (*C. difficile*) is the major infectious cause of nosocomial diarrhea and can cause prolonged hospital stays, renal failure, toxic megacolon, and death[1-3]. In 1995, the Society for Healthcare Epidemiology of America (SHEA) published a clinical position paper on *C. difficile*-associated disease (CDAD)[4]. Based on data from small, randomized, controlled studies showing no outcome-difference when comparing metronidazole and vancomycin, the 1995 position paper considered them equally effective; however, it stated, "metronidazole may be preferred to reduce the risk of vancomycin resistance among other organisms in hospitals".

Updated clinical practice guidelines for the management of CDAD in adults were published in 2010 by SHEA and the Infectious Diseases Society of America (IDSA)[5]. The 15-year interval between the two sets of recommendations was marked by dramatic changes in CDAD epidemiology and outcomes, with increases in prevalence, severity, and therapy resistance; emergence of hypervirulent strains may have contributed to these trends[6-8]. Additionally, new data suggested vancomycin might be superior for CDAD treatment in some cases. Zar *et al*[9] prospective, randomized, comparative efficacy study of metronidazole *vs* vancomycin demonstrated superiority of vancomycin for the treatment of severe CDAD. These results influenced the 2010 SHEA/IDSA guidelines that recommended vancomycin as first-line treatment for severe CDAD, while maintaining a recommendation for metronidazole in mild-moderate cases. These guidelines recommend treating an initial recurrence in the same manner as the initial episode, and a second recurrence with vancomycin in a tapering and/or pulsed regimen[5].

The 2010 SHEA /IDSA recommendations promote significant clinical practice changes. Since adherence to the guidelines may affect patient outcomes and infection control, we sought to determine adherence with the updated SHEA/IDSA CDAD guidelines at a tertiary care medical center.

MATERIALS AND METHODS

The Institutional Review Board of the University of Maryland Baltimore approved this study and waived the requirement for informed consent. All positive *C. difficile* stool tests (Quick Check A/B Toxin Assay; Wampole Laboratories, Princeton, New Jersey) in adults between May 2010 and May 2011 at the University of Maryland Medical Center were retrospectively identified. Medical charts were reviewed for demographics, clinical information, and adherence to CDAD guidelines.

Classifications defined in the updated 2010 SHEA/IDSA guidelines were used. These guidelines define mild-moderate CDAD as the presence of a white blood cell count $\leqslant 15000/mm^3$ and a serum creatinine level $\leqslant 1.5$ times the premorbid level. Conversely, severe CDAD is defined by the presence of a white blood cell count $\geqslant 15000/mm^3$ or a serum creatinine level $\geqslant 1.5$ times the premorbid level. Severe-complicated CDAD is defined by the presence of hypotension, shock, ileus, or megacolon. According to the guidelines, the correct treatment for mild-moderate CDAD is metronidazole 500 mg orally three times per day for 10-14 d. For treatment of severe CDAD, recommended treatment is oral vancomycin 125 mg four times per day for 10-14 d. For severe-complicated CDAD, the recommended treatment is oral vancomycin 500 mg four times per day in addition to intravenous metronidazole 500 mg every eight hours. If complete ileus exists, then rectal administration of vancomycin should be considered. Patients with a first recurrence are recommended to receive the same treatment as per their initial episode. For a second recurrence, vancomycin in a tapered and/or pulsed regimen is recommended.

Specific data collected included age, gender, disease severity as defined by the 2010 SHEA/IDSA guidelines, location of treatment (stratified into outpatient, hospital ward or intensive care unit), non-CDAD antibiotic treatment during the month preceding diagnosis, presence of immunosuppression, if the episode was a recurrence, 30 d mortality, and agent selection and dosage of CDAD treatment. CDAD episodes were classified as guideline adherent if treatment provided was with the correct agent(s) at the correct dosage(s). If one of these parameters was not in accordance with the guidelines, then the treatment regimen was deemed non-adherent. Partial adherence was defined as the patient receiving the correct antibiotic, but at the wrong dose. Patients stratified into adherent and non-adherent groups were compared to determine demographics and clinical factors predictive of guideline adherence. Logistic regression analysis was performed to assess the effect of multiple predictors on guideline adherence (SAS, version 9.2).

RESULTS

About 320 positive *C. difficile* stool tests were identified in 290 patients (average age 57.6 years, 43.1% female). Of the cases, 95.9% were in hospitalized patients and 15.6% were identified as a recurrence. Stratified by disease severity criteria set forth by the SHEA/IDSA guidelines, 42.2% of cases were mild-moderate, 48.1% severe, and 9.7% severe-complicated. Most (80.6%) of the severe-complicated cases met this criterion due to hypotension or shock. Full adherence with the guidelines was observed in 43.4% of cases; 65.9% for mild-moderate, which was significantly better than in severe (25.3%) and severe-complicated cases (35.5%) ($P < 0.001$) (Figure 1). Of the severe CDAD cases, 55.3% were managed incor-

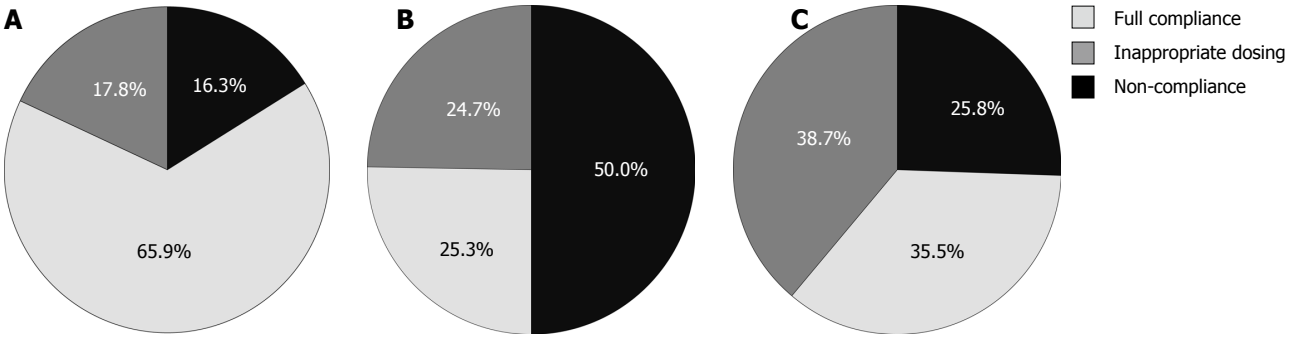

Figure 1 Rates of adherence with the 2010 the Society for Healthcare Epidemiology of America/the Infectious Diseases Society of America guidelines stratified by severity of *Clostridium difficile*-associated disease: (A) Mild-moderate, (B) Severe, and (C) Severe-complicated. Compliance was significantly better in mild-moderate *vs* severe or severe-complicated disease, $P < 0.001$.

Table 1 Comparison of demographics, disease severity, and other clinical factors between guideline adherent and guideline non-adherent groups *n* (%)

	Guideline Compliant, *n* = 139	Guideline Non-compliant, *n* = 181	Unadjusted *P* value	Adjusted *P* value
Demographics				
Mean ± SD, yr	56.8 ± 14.1	59.4 ± 16.2	0.13	
Female	61 (44.2)	77 (55.8)	0.81	
Disease severity				
Mild-moderate	89 (65.9)	46 (34.1)	< 0.001[1]	< 0.001[1]
Severe	39 (25.3)	115 (74.7)		
Severe-complicated	11 (35.5)	20 (64.5)		
Severe + severe-complicated	50 (27.0)	135 (73.0)		
Other factors				
Hospitalized	133 (43.3)	174 (56.7)	0.84	
ICU	60 (40.0)	90 (60.0)	0.24	
Prior antibiotics (< 30 d)	88 (39.1)	137 (60.9)	0.02	0.08
Recurrence	17 (34.0)	33 (66.0)	0.14	0.04
Immunosuppressed	57 (51.8)	53 (49.2)	0.03	0.49
30-d mortality	15 (41.7)	21 (58.3)	0.82	

[1]On unadjusted analysis, mild-moderate disease is compared to both severe and severe complicated disease. On adjusted analysis, mild-moderate disease is compared to the combination of severe and severe-complicated disease.

rectly with metronidazole. Partial adherence, where the correct drug was given at the incorrect dose, occurred in 17.8% of mild-moderate, 24.7% of severe, and 38.7% of severe-complicated cases (Figure 1).

On bivariate analysis (Table 1), factors significantly associated with adherence included disease severity, immunosuppression (IS), and documented receipt of antibiotics in the preceding 30 d. There was no difference in age, gender, hospitalization, ICU exposure, recurrence or 30-d mortality between adherent and non-adherent groups. IS patients were classified as mild-moderate more often than non-IS patients (60.0% *vs* 32.9%, $P < 0.001$). A multivariate model controlling for disease severity, prior antibiotics, IS, and recurrence status revealed significantly decreased adherence for severe/severe-complicated episodes (OR = 0.18, 95%CI: 0.11-0.30) and recurrent episodes (OR = 0.46, 95%CI: 0.23-0.95) but no significant difference for prior antibiotics or IS status.

DISCUSSION

Our results reveal poor overall adherence with the 2010 SHEA/IDSA guidelines for management of CDAD at a tertiary care academic medical center. Guideline adherence is worst in severe, severe-complicated, and recurrent CDAD. Our data suggests a lack of familiarity with current guidelines, as most providers continue to treat all initial episodes of CDAD with metronidazole, which was suggested as preferable by the 1995 SHEA clinical position paper on CDAD management. In fact, over half of our severe CDAD population, which should be treated with vancomycin, was incorrectly treated with metronidazole. This also explains the significantly improved adherence observed in mild-moderate patients whose treatment was not changed by the updated guidelines. We considered other possible causes of guideline non-adherence, such as the high cost of vancomycin and concern for vancomycin-resistance in other organisms, which has been shown to be significant in other nosocomial settings[10,11]. While the cost of branded oral vancomycin is approximately fifty-fold higher than oral metronidazole, our pharmacy routinely administers the generic intravenous formulation orally, which reduces the cost-difference dramatically[12], and makes cost concerns negligible. This finding also suggests an increased need for more intensive antibiotic stewardship, as not all incidences of non-adherence are likely due to knowledge. Antibiotic stewardship has been proposed as an effective method of increasing compliance at medical centers[13-15]. The exact impact of concerns over vancomycin resistance in other organisms on prescribing practices at our institution is unknown. We suspect this impact is small, as research on vancomycin use for CDAD has been conflicting with regards to rates of colonization and infection with resistant organisms[16-18].

Partial adherence with the guidelines, where the correct drug was chosen but an incorrect dosage was administered, occurred frequently as noted in Figure 1. The dosage of vancomycin chosen was often higher than recommended by the guidelines. While this is a form of

non-adherence, it may be appropriate, as patients given the recommended dosage (125 mg four times daily) can have low fecal levels during the first day of treatment[19]. Additionally, one would expect a higher dose to be equally effective and, given poor systemic absorption, little difference in side effects.

Interestingly, we found clusters of cases in specific wards of the hospital, such as the Trauma Unit or the Cancer Center, suggesting nosocomial spread of the infection, which is a major source of morbidity, mortality, and cost from the disease[20-22]. This finding highlights the need for improved hand hygiene and infection control measures such as contact precautions.

Two patient populations expose limitations of applying the SHEA/IDSA guidelines: immunosuppressed patients and patients with end-stage renal disease. As noted in our results, immunosuppressed patients were significantly more likely to have their disease severity classified as mild-moderate. This may reflect an inability to mount a severe-defining white blood cell count. The frequent incidence of neutropenia among cancer patients in particular suggests a limitation of applying the guidelines to this group. Similarly, the end-stage renal disease population, in whom serum creatinine fluctuations cannot be used as indicators of disease severity, present a problem with applying the SHEA/IDSA guidelines.

The present work has limitations. Our study was retrospective, took place in a single tertiary medical center, and 95.9% of CDAD cases were in hospitalized patients. Therefore, our results may not be applicable to community hospitals or outpatients. Additionally, our study was not powered to detect differences in 30-d mortality. Our institution was also using a toxin assay for diagnosis at the time of data collection, which is less sensitive than PCR[23,24]. Furthermore, the study by Zar *et al*[9] that was used to develop the treatment guidelines has had questions raised with regards to its methodology[25,26]. Further studies that directly survey providers about their treatment practices and measure the effects of guideline adherence on mortality and morbidity factors such as length of stay and complications of CDAD are warranted.

In conclusion, our results suggest that many providers are unfamiliar with the 2010 SHEA/IDSA *C. difficile* guidelines. Educational interventions and antibiotic stewardship may prove beneficial to improve adherence, and potentially patient outcomes.

COMMENTS

Background

Clostridium difficile (*C. difficile*) remains a major cause of nosocomial diarrhea, resulting in prolonged hospital stays, renal failure, toxic megacolon, and death. This also contributes to a significant economic burden on medical facilities across the globe. While the number of nosocomial infections increases, the correct treatment for this disease is of paramount importance.

Research frontiers

In 1995, the Society for Healthcare Epidemiology of America (SHEA) published guidelines that suggested that Metronidazole is the preferred agent for treatment of *C. difficile*. Recently, updated clinical practice guidelines for the management of *C. difficile* colitis have been published by SHEA and the Infectious Diseases Society of America (IDSA) suggesting that oral Vancomycin be preferred in cases of severe and severe-complicated disease, but adherence to these new guidelines is unclear at this time. In this study, the authors observe compliance to the new 2010 guidelines at a tertiary medical center.

Innovations and breakthroughs

Despite advances in health care sanitation technique, Clostridium infections continue to increase. In this study, the authors observed that compliance to the updated 2012 SHEA/IDSA guidelines is poor at the tertiary care hospital, suggesting a need for increased education and antibiotic stewardship for providers. The authors also identified specific areas that the guidelines fail to address clearly; end-stage renal disease patients and patients who are significantly immunosuppressed.

Applications

By recognizing poor compliance at our tertiary care facility, steps can be made to increase education and antibiotic stewardship at other facilities. In addition, the study suggests the guidelines should be updated to include the aforementioned patient populations with specific guidelines pertaining to their management.

Terminology

Compliance in the study is defined as using the proper dosage (both strength and frequency) in the proper duration for a specific *C. difficile*-associated diarrhea (CDAD) infection. The guidelines define mild to moderate CDAD as the presence of a white blood cell count $\leqslant$ 15000/mm^3 and a serum creatinine level $\leqslant$ 1.5 times the premorbid level. Conversely, severe CDAD is defined by the presence of a white blood cell count $\geqslant$ 15000/mm^3 or a serum creatinine level $\geqslant$ 1.5 times the premorbid level. Severe-complicated CDAD is defined by the presence of hypotension, shock, ileus, or megacolon.

Peer review

The authors report the results of a study conducted to assess adherence with the SHEA/IDSA guidelines for management of CDAD at a tertiary medical center. The study is well-designed, includes sufficient number of patients and the paper is well written. Despite the fact that the study is single-centered, and includes only hospitalized patients which reduces its generalizability (as mentioned by the authors), the results are considerable. This is a worthy study and appears of high clinical interest.

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BRIEF ARTICLE

Assessment of the diagnostic performance and interobserver variability of endocytoscopy in Barrett's esophagus: A pilot *ex-vivo* study

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Abstract

AIM: To investigate a classification of endocytoscopy (ECS) images in Barrett's esophagus (BE) and evaluate its diagnostic performance and interobserver variability.

METHODS: ECS was applied to surveillance endoscopic mucosal resection (EMR) specimens of BE *ex-vivo*. The mucosal surface of specimen was stained with 1% methylene blue and surveyed with a catheter-type endocytoscope. We selected still images that were most representative of the endoscopically suspect lesion and matched with the final histopathological diagnosis to accomplish accurate correlation. The diagnostic performance and inter-observer variability of the new classification scheme were assessed in a blinded fashion by physicians with expertise in both BE and ECS and inexperienced physicians with no prior exposure to ECS.

RESULTS: Three staff physicians and 22 gastroenterology fellows classified eight randomly assigned unknown still ECS pictures (two images per each classification) into one of four histopathologic categories as follows: (1) BEC1-squamous epithelium; (2) BEC2-BE without dysplasia; (3) BEC3-BE with dysplasia; and (4) BEC4-esophageal adenocarcinoma (EAC) in BE. Accuracy of diagnosis in staff physicians and clinical fellows were, respectively, 100% and 99.4% for BEC1, 95.8% and 83.0% for BEC2, 91.7% and 83.0% for BEC3, and 95.8% and 98.3% for BEC4. Interobserver agreement of the faculty physicians and fellows in classifying each category were 0.932 and 0.897, respectively.

CONCLUSION: This is the first study to investigate classification system of ECS in BE. This *ex-vivo* pilot study demonstrated acceptable diagnostic accuracy and excellent interobserver agreement.

Key words: Endocytoscopy; Barrett's esophagus; Dysplasia; Esophageal adenocarcinoma; Interobserver agreement

Core tip: The current gold standard for surveillance of esophageal adenocarcioma in Barretts's esophagus (BE) is endoscopic random biopsy and pathological diagnosis. Endocytoscopy (ECS) has the potential to provide a virtual histological diagnosis *in vivo* and in real-time. However, a major issue relates to that interpretation of cellular and nuclear images may be subject to similar interobserver variability associated with conventional histopathological diagnosis, and there have been no

reliable classification systems for the endocytoscopic diagnosis. We presented the first study to investigate classification system of ECS in BE. This *ex-vivo* pilot study demonstrated acceptable diagnostic accuracy and excellent interobserver agreement.

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INTRODUCTION

Recent advances in endoscopic imaging may lead to improved detection and facilitate therapy of dysplasia and esophageal adenocarcinoma (EAC) in Barrett's esophagus (BE)[1-5]. Histology is regarding as the gold standard for diagnosis of dysplasia and EAC[6,7], but sampling error and interobserver variability among gastrointestinal pathologists have been well described[8-11].

Endocytoscopy (ECS) is a probe-based technique which captures ultra-high magnified images of the epithelial surface, with the capability to discriminate cellular and subcellular features. ECS, thus, has the potential to provide a virtual histological diagnosis *in vivo* and in real-time. ECS has been investigated throughout the gastrointestinal tract for the identification of lesions in the esophagus[12-16], small intestine[17], and colon[18-21]. However, a major issue relates to the fact that interpretation of cellular and nuclear images may be subject to similar interobserver variability associated with conventional histopathological diagnosis[22,23]. To date, there have been no reliable classification systems for the endocytoscopic diagnosis of BE and Barrett's EAC. Accurate diagnosis based on a simple and reproducible classification system is warranted before ECS can be implemented into clinical practice.

Our aim was to develop simplified scheme for the classification of endocytoscopic images in BE and to evaluate its diagnostic performance and interobserver variability among experienced and inexperienced users of ECS in an *ex-vivo* setting.

MATERIALS AND METHODS

Patients and tissue specimens

ECS was performed *ex-vivo* on endoscopic mucosal resection (EMR) specimens obtained from patients undergoing endoscopic surveillance of BE at our institution. All EMR procedures were performed by a single endoscopist using the cap technique (EMR Kit; Olympus America, Center Valley, PA). Lesions targeted for EMR were endoscopically suspect areas, such as nodules or polyps, or dysplastic/neoplastic-appearing mucosa, such as irregular, friable, ulcerated, or villous-appearing mucosa, as seen under high-definition white-light imaging and narrow band imaging[24]. The study was approved by the Institutional Review Board and a written informed consent was obtained from all patients.

Endocytoscopy procedure

As soon as retrieved from the patient, the mucosal surface of each EMR specimen was immediately rinsed with 3-5 mL of 20% N-acetylcysteine to remove excess mucus, followed by the application of 1-1.5 mL of 1% methylene blue solution as contrast agent. *Ex-vivo* ECS imaging was performed using a flexible, catheter-type endocytoscope (XEC120, Olympus Medical Systems Co., Tokyo, Japan) which provides 1100 × magnification at a 120 μm × 120 μm field of view. The stained surface of each specimen was surveyed with the endocytoscope, and the area most representative of the endoscopically suspect lesion was identified. ECS imaging of the lesion was videotaped for approximately one minute.

Histopathology assessment

Histopathological assessment of the EMR specimens was performed according to the protocol in our BE unit, as previously published[25]. Patients had their pathological diagnosis of EMR specimens confirmed by at least two experienced gastrointestinal pathologists with expertise in Barrett-associated neoplasia.

ECS image analysis and classification

Following histopathological diagnoses of EMR specimens, the corresponding ECS videos and images were reviewed by investigators uninvolved in the subsequent blinded image assessment. To accomplish accurate correlation of endocytoscopic images with histological findings, we selected snapshots that were most representative of the histopathological findings for each specimen and matched final histopathological findings with their respective ECS images. With consideration to the previously proposed esophageal endocytoscopic atypia classification by Inoue *et al*[26], we classified the endocytoscopic images as follows (BEC; Barrett's EndoCytoscopy): (1) BEC1 - squamous epithelium; (2) BEC2-BE without dysplasia; (3) BEC3 - BE with dysplasia; and (4) BEC4-BE with EAC.

This classification scheme is based on the interpretation of ECS features (Figure 1): BEC1 images consist of cytoplasm-rich, rhomboid cells in a regular pattern; BEC2 images consist of increased cell numbers and different-sized nuclei/cells; BEC3 images consist of increased nucleus-cytoplasm ratio with dense chromatin and prominent nuclear fission; BEC4 images consist of cells of various sizes that are irregularly arranged, with blurred and enlarged nuclei. Two representative endocytoscopic images for each classification were selected for analysis of diagnostic performance and interobserver variability.

Diagnostic performance and interobserver variability

The diagnostic performance and inter-observer variabil-

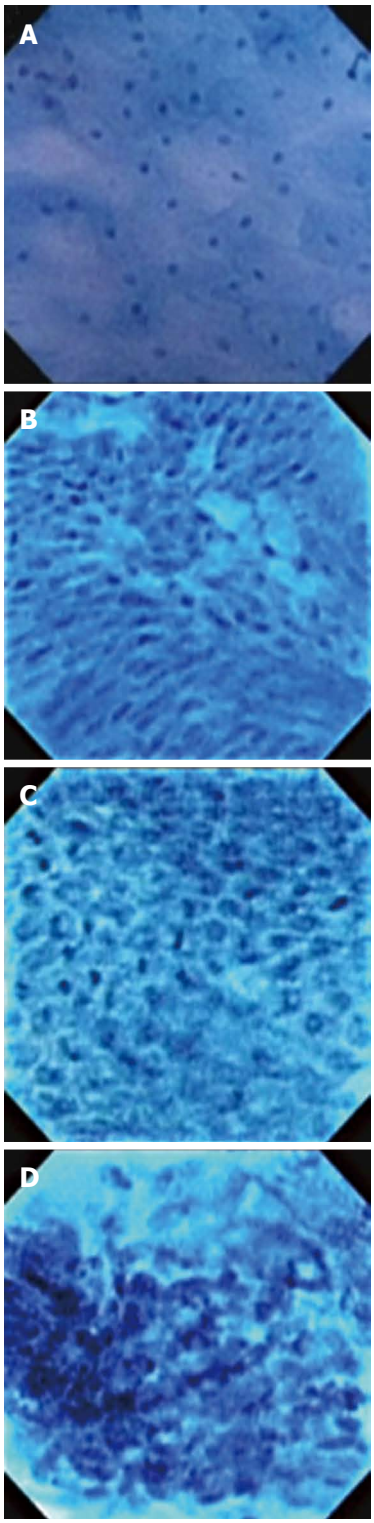

Figure 1 Classification of endocytoscopic images of Barrett's esophagus. A: Squamous epithelium (BEC 1) Cytoplasm-rich, rhomboid cells in a regular pattern; B: Barrett's esophagus without dysplasia (BEC 2) Increased cell number and different-sized nuclei/cells; C: Barrett's esophagus with dysplasia (BEC 3) Increased nucleus-cytoplasm ratio, and dense chromatin and nuclear fission are prominent; D: Esophageal adenocarcinoma (BEC 4) Cells of various sizes, irregularly arranged, with blurred and enlarged nuclei (magnification × 1125).

ity of the new classification scheme were assessed in a blinded fashion. Experienced physicians with expertise in both BE and ECS were provided with a brief 5-min presentation on the new classification, as shown in Figure 1. Inexperienced physicians consisted of clinical fellows (n = 22) in the Division of Gastroenterology and Hepatology with no prior exposure to ECS. They were provided with a 30 min presentation on ECS imaging and the new classification scheme. During the training session, fellows were presented with two non-study sets of pictures representative of each BEC classification for learning purposes. They were given the opportunity to ask questions and review the criteria. The training session and image classification by experienced and inexperienced physicians were conducted separately.

Immediately following the training session, participants were shown the randomly assigned unknown ECS pictures and asked to classify each image as: (1) BEC1; (2) BEC2; (3) BEC3; and (4) BEC4. The participants were blinded to patient history, endoscopic findings, and histopathological diagnoses. During the image classification session, the participants were not allowed to review previously seen images or to change their answers.

Statistical analysis

Data analysis was performed using the SPSS (Chicago, Illinois, United States) statistical software program. Classification accuracy, sensitivity, specificity, and positive and negative predictive values were calculated to assess diagnostic performance. Interobserver agreement was determined using intraclass correlation coefficient (ICC), which assesses agreement beyond chance among investigators. ICC was derived from a 2-way random effects model because both people effects and measures effects were random. An ICC of 0.4-0.75 indicates fair to good reliability, whereas an ICC greater than 0.75 shows excellent reliability.

RESULTS

A total of 20 patients were included in this study: squamous epithelium (n = 2), BE without dysplasia (n = 6), BE with dysplasia (n = 6), and BE with EAC (n = 6). A total of eight representative endocytoscopic images (two images per each classification) from different patients were utilized for this study. The overall classification accuracy for each category among experienced (n = 3) and inexperienced (n = 22) physicians were 100% and 99.4% for BEC1, 95.8% and 83.0% for BEC2, 91.7% and 83.0% for BEC3, and 95.8% and 98.3% for BEC4, respectively. If we combined BEC2 and BEC3 as diagnosis of BE, the classification accuracy would be 95.8%, even in ECS naive observers. The sensitivities, specificities, positive predictive values and negative predictive values for each category are shown in Table 1.

The interobserver agreements for the experienced and inexperienced physicians in classifying each category were 0.932 and 0.897, respectively. When a dichotomized category (BEC 1 and 2 *vs* BEC 3 and 4) was used, interobserver agreements for the experienced and inexpe-

Table 1 Diagnostic performance of endocytoscopy in Barrett's esophagus

	Classification	Sensitivity	Specificity	PPV	NPV
Experienced physicians (staff physician)	BEC 1	1.000%	1.000%	1.000%	1.000%
	BEC 2	1.000%	0.944%	0.857%	1.000%
	BEC 3	0.833%	0.944%	0.833%	0.944%
	BEC 4	0.833%	1.000%	1.000%	0.947%
Inexperienced physicians (clinical fellow)	BEC 1	0.977%	1.000%	1.000%	0.992%
	BEC 2	0.636%	0.894%	0.667%	0.881%
	BEC 3	0.705%	0.871%	0.646%	0.898%
	BEC 4	0.955%	0.992%	0.977%	0.985%
Both experienced and inexperienced (physicians staff physician and clinical fellow)	BEC 1	0.980%	1.000%	1.000%	0.993%
	BEC 2	0.680%	0.900%	0.694%	0.894%
	BEC 3	0.720%	0.880%	0.667%	0.904%
	BEC 4	0.940%	0.993%	0.979%	0.980%

PPV: Positive predictive value; NPV: Negative predictive value.

Table 2 Interobserver agreement of endocytoscopy in Barrett's esophagus

Classification	Staff physician (95%CI)	GI fellow (95%CI)
BEC 1-4	0.932 (0.794-0.985)	0.897 (0.784-0.973)
BEC 1 and 2 *vs* 3 and 4	0.851 (0.593-0.965)	0.581 (0.358-0.856)

GI: Gastrointestinal.

rienced physicians in classifying into this category were 0.851 and 0.581, respectively (Table 2).

DISCUSSION

Barrett's esophagus (BE) is a well-established precursor of esophageal adenocarcinoma (EAC) whose incidence is rising in Western countries. Patients with BE are therefore advised to undergo periodic surveillance to detect dysplastic mucosa and pre-cancerous lesions at an early stage at a time where intervention can be curative. The current gold standard for surveillance is periodic endoscopic random biopsy within the BE segment and pathological diagnosis. Due to inherent limitations of the gold standard, there have been considerable interests in advanced endoscopic imaging techniques to enhance dysplasia and EAC detection. Dysplasia can be patchy in distribution within the BE segment and sampling error can occur with random biopsy techniques. Novel imaging technologies that can reliably detect dysplasia or EAC in real-time would facilitate targeted biopsy and, hence, a reduction in sampling error.

Endocytoscopy (ECS) can provide real-time virtual histological images during endoscopic observation and potentially identify areas that harbor dysplastic or cancerous cells. However, interpretation of ECS images may be subject to similar interobserver variability associated with conventional histopathological diagnosis. A first step is to standardize ECS image criteria for accurate tissue diagnosis. To date, no studies have been conducted on the development and use of a classification system for endocytoscopic images in BE. In this study, we proposed a classification scheme based on ECS cellular and architectural features for categorizing Barrett's tissue, with the aim that the classification remains simple and easy to learn and adopt.

Overall, we had an acceptable classification accuracy for each Barrett tissue category when using our classification system and high accuracy was obtained for the differentiation of BE without dysplasia from dysplastic tissue among experienced observers. Although the number of percentage of accuracy among staff physicians appears low in BE with dysplasia, it is obvious that the small number of denominator is the reason. Our group of inexperienced observers (clinical fellows) classified squamous epithelium and EAC with high accuracy of 99.4% and 98.3%, respectively, and BE with and without dysplasia with acceptable accuracy of 83.0%. These results suggest our classification scheme is reliable and easy to learn. Interobserver agreement regarding both experienced and inexperienced groups was interpreted as excellent (ICC = 0.932 and 0.897, respectively). In classifying into two dichotomized category (BEC 1 and 2 *vs* BEC 3 and 4), interobserver agreement for the experienced physicians was still interpreted as excellent (ICC = 0.851) and interobserver agreement for the fellows showed good reliability (ICC = 0.581).

In this study, all the misdiagnoses of BE with dysplasia (BEC 3) were answered as non-dysplasia (BEC 2). It may imply that our criteria are similar to those of histological diagnosis. Misdiagnosis for non-dysplastic BE occurred in the inexperienced group, and all the misdiagnoses were answered as dysplasia. These facts probably reflect some of the dilemma that exists with pathological interpretation of non-dysplastic and dysplastic BE.

The reported diagnostic accuracy and interobserver agreement of ECS images should be interpreted with caution. In this structured pilot *ex-vivo* study, we intended to show a "classic" unambiguous image for each category and, thus, selected representative images. In BE, the microscopic epithelial changes that represent transition from metaplasia to dysplasia to cancer occur on a continuum. We did not assess how our classification performs near the margins of the transitions. The use of the representative images may maximize diagnostic

accuracy and interobserver agreement and minimize the correlation of the study findings with what will be observed during real-time use *in vivo*. We did not evaluate using "real-time" images correlated with biopsy results using our classification. Further study is warranted in real scanning to validate our classification. An additional study limitation is related to the *ex-vivo* nature of this preliminary study. The performance of *in-vivo* catheter-type ECS is clearly operator dependent. Challenges that lie ahead are the difficulty of maintaining a long, thin, flexible catheter onto the esophageal surface in stable position and in focus. During *in-vivo* observations, gastrointestinal motility may hinder collection of interpretable images. In one *in-vivo* study of ECS in BE[27], 76% of ECS images were recognized as poor quality. The study also had a high false-positive rate of 43%, resulting in both a sensitivity and positive predictive value of 42% in all image sequences. Conversely, an *in-vivo* feasibility ECS study for esophageal cancer conducted in Japan reported that clear and interpretable images were obtained in all cases, with the positive predictive value and the false-positive rate for esophageal malignancy being 94% and 6.3%, respectively[26]. Another *in-vivo* feasibility study also presented high quality images for interpretation[12]. Expertise in handling the ECS device could explain the difference in image quality obtained, and we believe the technical aspects can be overcome as already reported in the previous two *in-vivo* studies. A new endoscope-type ECS (XGIF-Q260EC1 and XCF-Q260EC1; Olympus) has recently been introduced and been reported to obtain more sensitive, ultra-magnified images[28-30]. The new ECS enables easy switch from a conventional endoscopic view to ultra-magnification endocytoscopic view by the press of a button at the top of the endoscope. The new device could reduce the technical burden of maintaining an ECS probe on a moving surface.

Our classification system does not differentiate between low grade (LGD) and high grade (HGD) dysplasia. It is well known the reproducibility of histopathological interpretation of LGD and HGD even among skilled pathologists is a challenge[10]. We still do not have definitive consensus about the management of LGD in BE, and management is individualized. It is clear that surveillance of any dysplastic lesions in BE segment is of importance given the established dysplasia-carcinoma sequence in EAC. In this study, we aimed to assess the potential of ECS in enhancing surveillance of dysplastic lesions in BE. We therefore proposed the two distinct criteria of dysplastic BE *vs* non-dysplastic BE instead of LGD and HGD.

In conclusion, we proposed a simple diagnostic classification system for ECS in BE. In this pilot *ex-vivo* study, acceptable accuracies regarding the diagnosis of squamous epithelium, non-dysplastic BE, dysplastic BE, and EAC were demonstrated. Interobserver agreement in classifying each category was interpreted as excellent, even among observers inexperienced in ECS. The applicability of the proposed classification scheme in the *in-vivo* setting remains to be seen.

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We thank Olympus America for providing the catheter-type endocytoscope for this pilot study.

COMMENTS

Background

Barrett's esophagus is a well-established precursor of esophageal adenocarcinoma, therefore it is very important to detect dysplastic pre-cancerous lesions at an early stage at a time where intervention can be curative. The current gold standard of endoscopic random biopsy has inherent limitations. There have been considerable interests in advanced endoscopic imaging techniques to enhance dysplasia detection.

Research frontiers

Novel imaging technologies that can reliably detect dysplasia or early esophageal cancer would facilitate targeted biopsy and, hence, a reduction in sampling error. Endocytoscopy can provide real-time virtual histological images during endoscopic observation and potentially identify areas that harbor dysplastic or cancerous cells and facilitate targeted biopsy.

Innovations and breakthroughs

A major issue relates to the fact that interpretation of cellular and nuclear images by endocytoscopy may be subject to similar interobserver variability associated with conventional histopathological diagnosis. To date, there have been no reliable classification systems for the endocytoscopic diagnosis of Barrett's esophagus and esophageal adenocarcinoma. In this study, the authors proposed a classification scheme based on endocytoscopy cellular and architectural features for categorizing Barrett's tissue, with the aim that the classification remains simple and easy to adopt. This is the first study to investigate classification system of endocytoscopy in Barrett's esophagus. In this study, the diagnostic performance and inter-observer variability of the new classification scheme were assessed in a blinded fashion by physicians with expertise in both Barrett's esophagus and endocytoscopy and inexperienced physicians with no prior exposure to endocytosocpy. Overall, the authors had an acceptable classification accuracy for each Barrett tissue category when using our classification system, and high accuracy was obtained for the differentiation of Barrett' s esophagus without dysplasia from dysplastic tissue among experienced observers. Interobserver agreement in classifying each category was interpreted as excellent among both experienced and inexperienced observers.

Applications

The results of this structured pilot *ex-vivo* study suggest that our classification scheme is reliable and easy to learn.

Peer review

The study to investigate classification system of endocytoscopy in Barrett's esophagus is very interesting. This *ex-vivo* pilot study demonstrated acceptable diagnostic accuracy and excellent interobserver agreement.

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BRIEF ARTICLE

Synergistic effect of interleukin-10-receptor variants in a case of early-onset ulcerative colitis

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Abstract

AIM: To investigated the molecular cause of very early-onset ulcerative colitis (UC) in an 18-mo-old affected child.

METHODS: We analysed the interleukin-10 (*IL10*) receptor genes at the DNA and RNA level in the proband and his relatives. Beta catenin and tumor necrosis factor-α (TNFα) receptors were analysed in the proteins extracted from peripheral blood cells of the proband, his relatives and familial adenomatous polyposis (FAP) and PTEN hamartoma tumor syndrome (PHTS) patients. Samples were also collected from the proband's inflamed colorectal mucosa and compared to healthy and tumour mucosa collected from a FAP patient and patients affected by sporadic colorectal cancer (CRC). Finally, we examined mesalazine and azathioprine effects on primary fibroblasts stabilised from UC and FAP patients.

RESULTS: Our patient was a compound heterozygote for the *IL10RB* E47K polymorphism, inherited from his father, and for a novel point mutation within the *IL10RA* promoter (the -413G->T), inherited from his mother. Beta catenin and tumour necrosis factor α receptors-Ⅰ (TNFRI) protein were both over-expressed in peripheral blood cells of the proband's relatives more than the proband. However, TNFRII was over-expressed only in the proband. Finally, both TNFα-receptors were shown to be under-expressed in the inflamed colon mucosa and colorectal cancer tissue compared to healthy colon mucosa. Consistent with this observation, mesalazine and azathioprine induced, in primary fibroblasts, *IL10RB* and TNFRII over-expression and TNFRI and TNFα under-expression. We suggest that β-catenin and TNFRI protein expression in peripheral blood cells could represent molecular markers of sub-clinical disease in apparently healthy relatives of patients with early-onset UC.

CONCLUSION: A synergistic effect of several variant alleles of the *IL10* receptor genes, inherited in a Mende-

lian manner, is involved in UC onset in this young child.

Key words: Inflammatory bowel disease; Ulcerative colitis; Interleukin 10 receptors; Tumour necrosis factor α receptors; Beta catenin

Core tip: We identified a novel point mutation within the interleukin-10 (*IL10*) receptor genes promoter (the -413G->T), associated with mRNA under-expression. We propose that this mutation has a synergistic effect with other variant alleles of *IL10* receptor genes in very-early ulcerative colitis (UC) onset in this young child. β-catenin and tumour necrosis factor α receptors-I (TNFRI) protein were both over-expressed in peripheral blood cells of proband relatives, whereas TNFRII was over-expressed only in the proband. We suggest that β-catenin and TNFRI protein expression could represent molecular markers of sub-clinical disease in apparently healthy relatives of patients with early-onset UC.

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INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic relapsing inflammatory disorders thought to result from an inappropriate and continuing inflammatory response to commensal microbes in a genetically susceptible host[1]. Crohn's disease (CD) and ulcerative colitis (UC) are the two main clinicopathological subtypes of IBD, common in developed countries, affecting the quality of life of approximately 1.4 million individuals in the United States and 2.2 million people in Europe[2-4].

Accumulating data suggest that these disorders result from an inappropriate inflammatory response to intestinal microbes in a genetically susceptible host[5]. Active IBD is defined as an infiltration of the lamina propria by innate immune cells (neutrophils, macrophages, dendritic and natural killer T cells) and adaptive immune cells (B and T cells). Increased numbers and activation of these cells in the intestinal mucosa enhance local levels of tumour necrosis factor-α (TNFα) and several pro-inflammatory interleukins (IL)[5-8].

Genome-wide association studies (GWAS) have been successful in IBD, identifying 99 non-overlapping genetic risk loci, including 28 that are shared between CD and UC[9,10]. Analyses of the genes and genetic loci implicated in IBD show several pathways that are crucial for intestinal homeostasis, including barrier function, epithelial restitution, microbial defence, innate immune regulation, reactive oxygen species generation, autophagy, adaptive immunity regulation, endoplasmic reticulum stress and metabolic pathways associated with cellular homeostasis. Early studies have suggested the existence of both protective and predisposing alleles[11]. Again, many genetic changes might affect genetic regions other than coding regions, indicating that allele-specific gene-expression changes contribute to the disease risk[12].

The relative importance of each individual pathway in the pathogenesis of IBD has not been determined. There is enthusiasm for a model in which mucosal inflammation results from defective activity of Treg cells. In this model, effector T cells that react to the microbial flora or other GI antigens are kept in check by a population of regulatory cells; defects in these cells lead to GI inflammation. IL10 production by Treg cells appears to be required for suppression of colitis[13].

A recent study has demonstrated that IBD with an early onset can be monogenic. Mutations in *IL10* or its receptor lead to a loss of IL10 function and cause severe intractable enterocolitis in infants and small children[14].

IL10R consists of two α (*IL10RA*) and two beta (*IL10RB*) molecules. *IL10RA* and *IL10RB* genes have been mapped on chromosomes 11q23.3 and 21q22, respectively, and many single-nucleotide polymorphisms (SNPs) have been identified[15]. Recently, Moran *et al*[16] identified *IL10Rs* polymorphisms that confer risk for developing very early-onset IBD. Each novel, nonsynonymous SNP was identified only in the heterozygous state, and none of the resulting amino acid changes were predicted to be deleterious by SIFT or Polyphen.

The aims of this work were to clarify the molecular basis of UC in an 18-mo-old affected child. To this aim, we investigated the pathogenetic mechanisms of IL10 pathway alteration in the onset of UC in the proband, and we clarified the molecular changes associated with them. Moreover, we propose β-catenin and tumour necrosis factor α receptors- I (TNFRI) as molecular biomarkers of subclinical disease among apparently healthy family members of the index case. Finally, we have investigated the effect of mesalazine and azathioprine, the main pharmacological therapy used for IBD treatment, on the expression of IL10 receptors, TNFα and TNFα receptors.

MATERIALS AND METHODS

Patients

The proband, exhibiting UC, was referred by paediatric gastroenterologists to the laboratory for genetic analysis. He was admitted to the hospital for bloody diarrhoea, asthenia, fever and a severe anaemia (haemoglobin 3.7 g/dL). He underwent upper and lower GI endoscopy. The upper GI endoscopy did not reveal any macroscopic and/or microscopic sign of disease. Ileocolo-

noscopy showed a severe ulcerative pancolitis, (E4-S1) according to the Paris classification[17]. The colonoscopic grade of inflammation was characterised by the presence of marked erythema, absent vascular pattern, friability erosions, associated with spontaneous bleeding and ulcerations, suggesting a grade 3 according to the Mayo endoscopic score[18]. A severe grade of inflammation was confirmed histologically by the diffuse presence of a large number of neutrophilic leukocytes (> 50/HPF) with crypt abscesses and significant acute inflammation with ulcerations in lamina propria. The presence of granulomas was excluded at any colonic levels, as well as at level of the distal ileum.

The child was treated with blood transfusions, antibiotics and steroid therapy without improvement. A rescue therapy with cyclosporine followed by mesalazine and azathioprine was then started. His following clinical history was characterised by relapsing-remitting symptoms and by the lack of response to drugs. The proband's mother referred episodes of bloody diarrhoea, but she refused colonoscopy.

Blood samples from proband and healthy family members were collected at the same hospital as the patient. Normal colorectal mucosa and colorectal cancer tissues were sampled from patients with FAP or sporadic colon cancer operated on the "Istituto Nazionale dei Tumori" in Naples.

Samples from all subjects who participated in the study were collected after being granted authorisation from the "Comitato etico per le attività Biomediche - Carlo Romano" of the University of Naples Federico Ⅱ, with protocol number 120/10. Such authorisation is given only once the study has received ethical approval, and participants' informed and written consent has been obtained.

Molecular analysis of IL10RA and IL10RB messenger

Reverse transcription polymerase chain reaction of *IL10RA* and *IL10RB* of full length coding regions: Total RNA was extracted from 3 mL of peripheral blood cells of the UC patient and his healthy family members, using Trizol reagent (Invitrogen, Life Technologies, CA), cDNA was synthesised and 1 μL of the cDNA was amplified by reverse transcription polymerase chain reaction (RT-PCR) as previously described[19], using the following pairs of oligonucleotides: *IL10RA*-5'UTR-FP/*IL10RA*-3'UTR-RP; *IL10RB*-5'UTR-FP/*IL10RB*-3'UTR-RP. Two fragments of 2023 bp and 1197 bp, respectively, were produced. The PCR products were analysed on a 1% agarose gel in a tris-acetic acid (TAE)-EDTA standard buffer, and visualised by ethidium bromide staining (Table 1).

Sequence analysis of *IL10RA* and *IL10RB* mRNA: Sequence analysis of *IL10RA* and *IL10RB* full length coding regions was performed on amplified fragments from the cDNA of the proband and his healthy family members, using the following primer pairs, localised inside these regions: *IL10RA*-5'UTRb-FP; *IL10RA*-3'UTRb-RP; *IL10RA*-3cFP; *IL10RA*-4cRP; *IL10RA*-6cFP; *IL10RA*-7cRP; *IL10RB*-5'UTRb-FP; *IL10RB*-3'UTRb-RP; *IL10RB*-4cFP; *IL10RB*-5cRP (Table 1). The analysis was performed in a 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). For nucleotide numbering, the first A of the initiator ATG codon is nucleotide +1 of *IL10RA* and *IL10RB* mRNA sequences [GenBank Accession numbers: NM_001558.3 and NM_000628.3, respectively]; all oligonucleotides were obtained with primer-BLAST Software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Real time RT-PCR quantification analysis: Real time PCR quantification analysis was performed for *IL10RA* and *IL10RB* messengers. The relative expression was calculated with the comparative Ct method. Patient numbering corresponds to that adopted in Figure 1A. Three millilitres of peripheral blood cells from the UC patient, his healthy family members and 8 healthy subjects were pelleted after erythrocyte lysis and resuspended in Trizol reagent. The mean value across all of the healthy samples ($H_{1\text{-}8}$) was used as a calibrator to measure the relative expression. *IL10RA* and *IL10RB* mRNA quantification was carried out by amplifying fragments spanning the junctions between exons 3-4, for *IL10RA* messenger and exons 4-5 for *IL10RB* messenger, compared to the glucuronidase transcript fragment, using the oligonucleotides described above: *IL10RA*-3cFP/*IL10RA*-4cRP; *IL10RB*-4cFP/*IL10RB*-5cRP (Table 1). The quantitative real time assays were performed using the iCycler iQ Real Time Detection System BIO-RAD as previously described[19].

Molecular analysis of IL10RA gene

Genomic PCR and sequencing: Genomic DNA was extracted from 3 mL of peripheral blood cells of UC patient, using Nucleon BACC2 Kit (Amersham Biosciences). Genomic PCR and sequencing of all exons was performed for *IL10RA* gene, using oligonucleotides complementary to intronic neighbouring boundary regions of each exon, described in Table 1. The GenBank Accession number of *IL10RA* genomic sequence is: (NC_ 000011.9/gi:224589802). Mutational analysis of *IL10RA* promoter region, from bp -2159 to bp +1, was performed by PCR and sequencing. This region was amplified into three overlapping fragments of 788, 782 and 788 bp in molecular weight, respectively, using the following primer pairs: *IL10RA*p1-FP/*IL10RA*p1-RP; *IL10RA*p2-FP/*IL10RA*p2-RP; *IL10RA*p3-FP/*IL10RA*p3-RP (Table 1).

Amplification refractory mutation-PCR of the -413G->T IL10-RA promoter mutation: We set up an amplification refractory mutation-PCR (ARMS-PCR) reaction to analyse 200 DNA extracted from blood samples of control subjects apparently healthy, for the -413G->T promoter mutation identified in the UC proband and his mother.

Table 1 Oligonucleotide sequences

	RT-PCR of IL10RA and IL10RB of full length coding regions	
IL10RA-5′UTR-FP:	GTCCCAGCCCAAGGGTAG	[NM_001558.3; start: + 5]
IL10RA-3′UTR-RP:	CACCCACATACCCTGCACTA	[NM_001558.3; start: + 2027]
IL10RB-5′UTR-FP:	GTCGTGTGCTTGGAGGAAG	[NM_000628.3; start: + 57]
IL10RB-3′UTR-RP:	GTGGCTAAGTCCAGGGTCTG	[NM_000628.3; start: + 1223]
	Sequence analysis of IL10RA and IL10RB messenger/real time RT-PCR quantification analysis	
IL10RA-5′UTRb-FP:	TCAGACGCTCATGGGACA	[NM_001558.3; start: + 132]
IL10RA-3′UTRb-RP:	CCCAGTGGACTTGCAGAAA	[NM_001558.3; start: + 1938]
IL10RA-3cFP:	AACTGGACCGTCACCAACAC	[NM_001558.3; start: + 405]
IL10RA-4cRP:	AATCTTCCCGAGGATGAAGC	[NM_001558.3; start: + 506]
IL10RA-6cFP:	AGCTACCCAGTGTCCTGCTC	[NM_001558.3; start: + 871]
IL10RA-7cRP:	CAAAAAGGCCTCCTCATCAA	[NM_001558.3; start: + 983]
IL10RB-5′UTRb-FP:	CATGGCGTGGAGCCTT	[NM_000628.3; start: + 99]
IL10RB-3′UTRb-RP:	GATGGTCTTGGCCCTTGTT	[NM_000628.3; start: + 1177]
IL10RB-4cFP:	GTGCAATACTGGAAAAACGGT	[NM_000628.3; start: + 565]
IL10RB-5cRP:	CCCTCGAACTTGAACACAATAA	[NM_000628.3; start: + 678]
	Genomic PCR and sequencing	
*IL10RA*p1-FP:	GCGGTTTGAGGCTCAGC	[NC_ 000011.9; start: + 117856447]
*IL10RA*p1-RP:	CAAGACGGAGGCTGAGGA	[NC_ 000011.9; start: + 117857234]
*IL10RA*p2-FP:	CTAGCAGGGGAAGAGCAGC	[NC_ 000011.9; start: + 117855574]
*IL10RA*p2-RP:	AACCTTCGTCTCCCAGGTTC	[NC_ 000011.9; start: + 117856355]
*IL10RA*p3-FP:	TGAGCCAAGTGACACAGAGG	[NC_ 000011.9; start: + 117855023]
*IL10RA*p3-RP:	TTGAACATATACCCTGCTGAAGAG	[NC_ 000011.9; start: + 117855810]
IL10RA-1FP:	CTGTCAGTCCCAGCCCAA	[NC_ 000011.9; start: + 17857104]
IL10RA-1RP:	TCTCCACTGGATGGAGAACTTTA	[NC_ 000011.9; start: + 117857327]
IL10RA-2FP:	TTGGTAAAATTGGGGTCATCA	[NC_ 000011.9; start: + 117859029]
IL10RA-2RP:	GCCCTCAGGCACTCACTTC	[NC_ 000011.9; start: + 117859328]
IL10RA-3FP:	AAGCTCGTTTCCAGTGCCTA	[NC_ 000011.9; start: + 117860120]
IL10RA-3RP:	GGCAGACATGGTGAGCTATG	[NC_ 000011.9; start: + 117860439]
IL10RA-4FP:	ACAAACCTGTGGCCAAGTTT	[NC_ 000011.9; start: + 117863822]
IL10RA-4RP:	CACACAAGGGTGCTTCCAG	[NC_ 000011.9; start: + 117864202]
IL10RA-5FP:	ATCACCTCTAAAGGCCCACC	[NC_ 000011.9; start: + 117864629]
IL10RA-5RP:	GGATGCAGAGCTATGTGAAGC	[NC_ 000011.9; start: + 117864993]
IL10RA-6FP:	TTTCATGGGACCAGAGTCCT	[NC_ 000011.9; start: + 117866223]
IL10RA-6RP:	CTGGCTGGGAGGAAAAGAG	[NC_ 000011.9; start: + 117864993]
IL10RA-7.1FP:	GCTCTCCTCCTGGGCCT	[NC_ 000011.9; start: + 117869338]
IL10RA-7.1RP:	CGGCCCTCAGAGTTTTGA	[NC_ 000011.9; start: + 117869854]
IL10RA-7.2FP:	ACCTGGGAGCAACAGGTG	[NC_ 000011.9; start: + 117869775]
IL10RA-7.2RP:	CGTGCCTAACTTCTGCCC	[NC_ 000011.9; start: + 117870445]
	ARMS PCR of the -413G->T IL10-RA promoter mutation	
IL10RA-ARMS-FP-N:	CCGGCACGCCAGGCAAAAGCGGCTCGGTCG	[NC_ 000011.9; start: + 117856738]
IL10RA-ARMS-FP-M:	CCGGCACGCCAGGCAAAAGCGGCTCGGTCT	[NC_ 000011.9; start: + 117856738]
IL10RA-ARMS-RP:	GCCTCCAGTGCCTTCGGATCAA	[NC_ 000011.9; start: + 117856897]
	Gene copy number quantification of IL10RA gene	
IL10RA-4cFP:	TCCTCGGGAAGATTCAGCTA	[NM_001558.3; start: + 493]
IL10RA-4c2RP:	TGCGAATGGCAATCTCATAC	[NM_001558.3; start: + 594]
IL10RA-7cFP:	ACTGAAGAGCCCCAGTTCCT	[NM_001558.3; start: + 1065]
IL10RA-7c2RP:	GCTGTCTGTGCTATTGCTGC	[NM_001558.3; start: + 1187]

RT-PCR: Reverse transcription polymerase chain reaction; IL10: Interleukin-10.

This ARMS reaction was performed with following oligonucleotide primers: *IL10RA*-ARMS-FP-N; *IL10RA*-ARMS-FP-M; *IL10RA*-ARMS-R (Table 1).

Gene copy number quantification of *IL10RA* gene: For the genomic quantification of *IL10RA* gene, specific amplified fragments were compared to a fragment of the exon 15 of *MUTYH* gene. For *IL10RA* specific quantification, two short fragments, one inside exon 4 and the other inside exon 7, were amplified, using the following primer pairs: *IL10RA*-4cFP/*IL10RA*-4c2RP; *IL10RA*-7cFP/*IL10RA*-7c2RP (Table 1). Patient numbering corresponds to that adopted in Figure 1A.

In silico analysis

In silico analysis of the -413G->T point mutation was performed using the Patch 1.0 software. Patch is a pattern-based program for predicting transcription factor binding sites (TFBS) in DNA sequences. It uses the set of binding sites from TRANSFAC® Public 6.0 and is free online available at the web site: http://www.biobase-international.com/.

β-catenin, TNFRI and TNFRII protein analysis in peripheral blood cells of UC patients

Western blotting assay of β-catenin, TNFRI and TNFRII proteins: Total protein was extracted from 3 mL

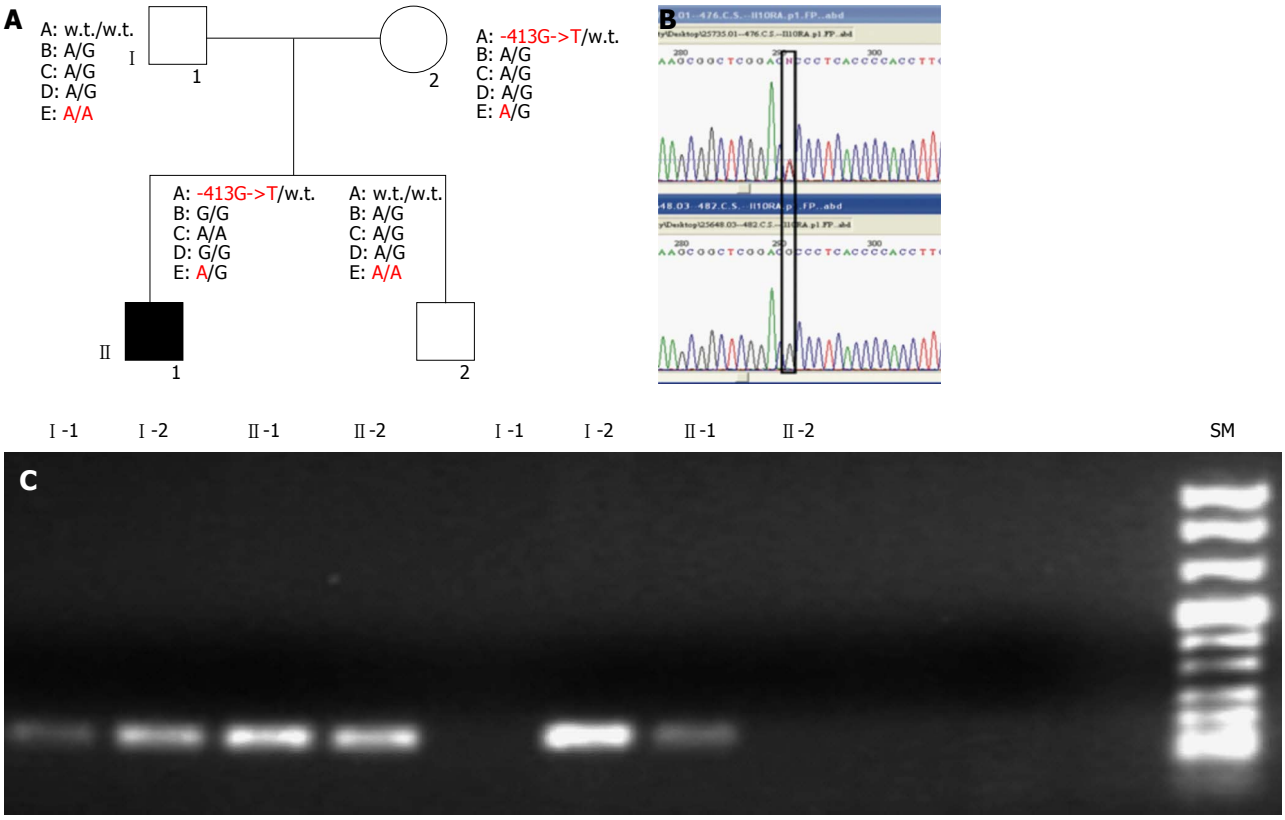

Figure 1 Molecular characterisation of variant alleles within *interleukin-10* receptor genes in the inflammatory bowel diseases family members. A: Pedigree of the inflammatory bowel diseases (IBD) family and genomic single-nucleotide polymorphisms identified: interleukin-10 (*IL10*) *RA*: - 413G->T (A); *IL10RA*-rs.: 2256111 Esone 4 c.549A->G (p.153Ala->Ala) (B); *IL10RA*-rs.:2229113 Esone 7 c.1051A->G (p.351Arg->Gly) (C); *IL10RA*-rs.:9610 3'UTR c.2543G->A (D); *IL10RB*-rs.: 2834167 Esone 1 c.139G->A (p.47 Lys ->Glu) (E); B: Sequence analysis of *IL10RA* promoter region. Sequence analysis was performed on amplified fragments from gDNA of the patients. Reported here are the electropherogram around the identified mutation - 413G->T. The specific mutated nucleotide is shown within the black box; C: Gel-electrophoresis of the amplification refractory mutation-polymerase chain reaction performed for the - 413G->T *IL10RA* promoter mutations. Patient numbering corresponds to that adopted in the shown above pedigree.

of peripheral blood cells (approximately 5-7 × 10^3/mL cells) using Trizol reagent (Invitrogen, Life Technologies, CA) following the manufacturer's instructions. Concentrations were determined and Western blotting assay was performed as previously described[19]. The primary antibody against amino-terminal β-catenin was from Cell Signaling Technology (Beverly, MA). Primary antibodies against TNFRI and TNFRII were from R&D System (R and D System, Minneapolis). The antibody against actin was from Santa Cruz (Santa Cruz, CA). H1-5 and H6-10 are mixes of healthy subjects. PHTS and FAP are two patients affected by PTEN hamartoma tumour syndrome and adenomatous polyposis coli syndrome, respectively. I -1, I -2, II -1 and II -2 are UC family members as reported in Figure 1A.

Real time PCR quantification analysis of *COX2* mRNA: Real time PCR quantification analysis was performed for *COX2* messengers. Relative expression was calculated with the comparative Ct method and normalised against the Ct of Glucuronidase (GUS) mRNA. The quantitative RNA real time assays were performed as described before. To better normalise the healthy values, we used three blood mixes as controls, each containing five samples collected from healthy subjects, for a total of fifteen controls. H1-5, H6-10, H11-15 are mixes of healthy subjects. Hm is the mean value among all healthy samples used as calibrator to measure the relative expression. Patient numbering corresponds to that adopted in Figure 1A.

β-catenin, TNFRI and TNFRII proteins expression in colorectal mucosa

Western blotting assay of β-catenin, TNFRI and TNFRII proteins: Total protein was extracted from the injured colorectal mucosa of the IBD proband and from healthy and tumour mucosa collected from patients affected by FAP and sporadic colorectal cancer using Trizol reagent (Invitrogen, Life Technologies, CA) following the manufacturer's instructions. Western blotting analysis of β-catenin (amino-terminal antigen), TNFRI and TNFRII was performed as previously described.

Incubation with mesalazine and azathioprine of established colon fibroblast culture: Samples of colorectal mucosa from IBD proband and one FAP patient were washed three times in PBS containing 300 U/mL penicillin, 300 μg/mL streptomycin, and 2.5 μg/mL amphotericin B (all from Gibco BRL, Karlsruhe, Germany), finely minced with scissors (tissue pieces of approximately 30 mm^3) and digested in 2 mL 0.1% collagenase II (Boehringer Man-

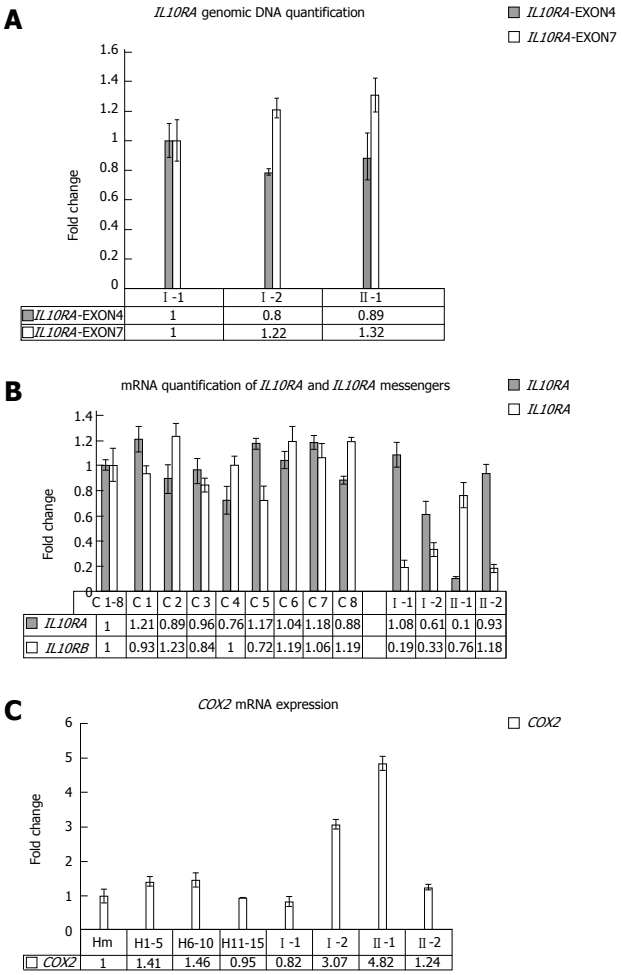

Figure 2 Real time polymerase chain reaction analysis of *interleukin-10 receptors* and *COX2* performed on peripheral blood cells. A: Copy number quantification of interleukin-10 *(IL10)* gene. Real time polymerase chain reaction (PCR) quantification analysis was performed for *IL10RA*. *IL10RA*-exon4: Amplified fragment at the boundaries of exon 4 and IVS4 of the gene; *IL10RA*-exon7: Amplified fragment at the boundaries of exon 7 and IVS7 of the gene; Patient numbering corresponds to that adopted in the pedigree shown in Figure 1A. B: Real time PCR quantification analysis of *IL10RA* and *IL10RB* mRNA. Real time RT-PCR quantification analysis was performed for *IL10RA* and *IL10RB* mRNA. C1-8: Mean value between all healthy samples used as calibrator to measure the relative expression; C1 to C8: Healthy subjects. Patient numbering corresponds to that adopted in the pedigree shown in Figure 1A. C: Real Time PCR quantification analysis of *COX2 messenger.* H1-5, H6-10, H11-15: Mixes of healthy subjects; Hm: Mean value between all healthy samples used as calibrator to measure the relative expression; Patient numbering corresponds to that adopted in the pedigree shown in Figure 1A.

nheim, Mannheim, Germany) in DMEM-15% FBS for 2 h at 37 ℃, 5% CO_2. The cell suspension was then collected by centrifugation, washed twice with serum-free DMEM medium, and subsequently cultured for 7 d in DMEM-15% FBS/CHANG C medium (1:1), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B (all from Gibco BRL, Karlsruhe, Germany). Primary fibroblasts from IBD and FAP patients were stabilised, cultured on plates, and incubated with mesalazine (30 mmol/L) and azathioprine (30 mmol/L) for 12 h, alternatively. A combination of real time PCR of *IL10* receptors and Western blotting analysis of TNFα and TNFα receptors were performed as previously described.

RESULTS

Variant alleles of the IL10 receptor genes act in a synergistic manner in the onset of UC

Molecular screening of *IL10RA* and *IL10RB*, performed on the proband and his relatives, revealed the presence of multiple SNPs in the patient, inherited from his parents, as shown in Figure 1A.

Specifically, the proband was heterozygous for the *IL10RB* E47K polymorphism (rs2834167, A/G genotype), inherited from his father, described to be associated with a low level of specific mRNA expression (to the A allele). As shown in Figure 1, he was also carrier of an *IL10RA* promoter point mutation (the -413G->T point mutation), inherited from his mother and not previously described in literature. In silico analysis of this mutation, performed using the Patch 1.0 software, shows that it alters a binding site for the Sp1 transcription factor. This genomic variant represents a specific mutation of this IBD family because it was not identified in 200 healthy subjects. The proband's father and his brother were both homozygous for *IL10RB* E47K polymorphism (rs rs.:2834167 A/A genotype; 47K/K), whereas his mother was heterozygous A/G. Only the proband and his mother were carriers of the -413G->T point mutation identified in the promoter region of the *IL10RA* gene. For the following SNPs of *IL10RA*, the rs2256111, localised in the exon 4 (c.549A->G; p.153Ala->Ala), the rs.:2229113, localised in the exon 7 (c.1051A->G; p.351Arg->Gly) and the rs.:9610, localised in the 3'UTR (c.2543G->A), the proband was homozygous G/G, G/G and A/A, respectively. These tree polymorphisms were A/G heterozygous in all other family members (Figure 1A). Using DNA real-time PCR for gene dosage of *IL10RA* gene, we ruled out the presence of intragenic or whole gene deletion (Figure 2A).

IL10 receptor variants are associated with mRNA under-expression

Associated with these genomic variants, we observed a under-expression of *IL10RA* and *IL10RB* mRNA in the proband compared to the average values of 8 healthy subjects, which segregates with each specific variant among the family members. In fact, as revealed by mRNA real-time quantification of both mRNAs of *IL10* receptors shown in Figure 2B, only the proband and his mother, carriers of the -413G->T promoter point mutation, showed a decrease in *IL10RA* mRNA. In contrast, the proband's father and his brother, both homozygous A/A for the *IL10RB* E47K polymorphism, show very low levels of *IL10RB* mRNA expression (fold change of approximately 0.19 and 0.18, respectively), whereas the proband and his mother, who were heterozygous A/G for this polymorphism, showed approximately 50% mRNA expression of the *IL10RB* compared to the mean value across eight healthy samples used as a calibrator (fold change of approximately 0.5 and 0.7 for the proband's mother and the proband himself, respectively). Furthermore, only the proband and his mother showed

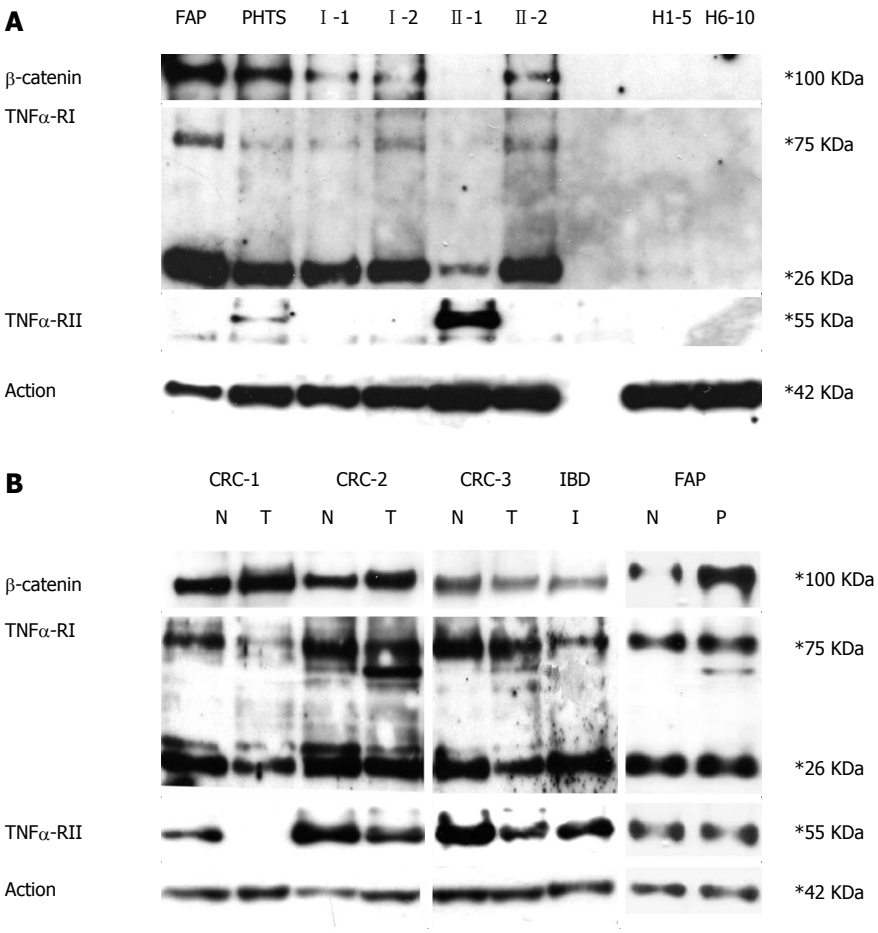

Figure 3 β-catenin, tumour necrosis factor α receptors-I and II protein expression performed on peripheral blood cells and colon mucosa. A: Western blotting assay of β-catenin tumour necrosis factor α receptors-I (TNFRI) and TNFRII performed on protein extracts from peripheral blood cells. Familial adenomatous polyposis (FAP): Patient affected by adenomatous polyposis coli; PHTS: Patient affected by PTEN hamartoma tumour syndrome; I-1, I-2, II-1, II-2: Patient numbering corresponds to that adopted in the pedigree shown in Figure 1A. H1-5, H6-10: mixes of healthy subjects; B: Western blotting assay of β-catenin TNFRI and TNFRII performed on protein extracts from colon mucosa. FAP: Patient affected by adenomatous polyposis coli; colorectal cancer (CRC)1, CRC2, CRC3: Patients affected by sporadic colorectal mucosa; inflammatory bowel diseases (IBD): Affected proband; N: Healthy colon mucosa; T: Colon tumour; P: Colon polyp; I: Inflamed colon mucosa.

COX2 overexpression, analysed in peripheral blood cells (Figure 2C).

Alteration of WNT/β-catenin pathway and TNFα receptors expression in the UC patient

As shown in Figure 3A, β-catenin and TNFRI protein were both over-expressed in the peripheral blood cells of the proband's relatives more than the proband. In contrast, TNFRII was over-expressed only in the proband. None of these proteins were detectable in healthy controls. When investigated in colon mucosa, both TNFα receptors were observed to be under-expressed in the inflamed colon mucosa and colorectal cancer compared to healthy colon mucosa. In the FAP patient, normal colon mucosa and polyps express TNFα receptors at the same level. Furthermore, as expected, β-catenin expression is much higher in the polyp than in normal mucosa. (Figure 3B)

Effects of mesalazine and azathioprine on primary fibroblasts

Finally, we show that after incubation with mesalazine and azathioprine of primary fibroblasts of the proband and of a FAP patient, drugs induce *IL10RB* mRNA and TNFRII protein over-expression, whereas TNFRI protein was under-expressed. A decrease of TNFα expression was also observed after incubation with azathioprine but not with mesalazine only in the IBD patient. Fibroblasts isolated from an FAP patient did not show any signal for TNFα hybridisation in our experimental conditions (Figure 4).

DISCUSSION

A recent study demonstrated that mutations in *IL10* or its receptor lead to a loss of IL10 function and cause severe intractable enterocolitis in infants and small children[20,21]. In another approach to determining the genetic basis for these disorders, Moran *et al*[16] identified risk SNPs for very early onset IBD. Two SNPs, rs2228054 and rs2228055, were frequently found in the heterozygous state among IBD patients and inherited as a haplotype. The authors propose that the conferred risk may be due to one or both SNPs. Alternatively, the increased

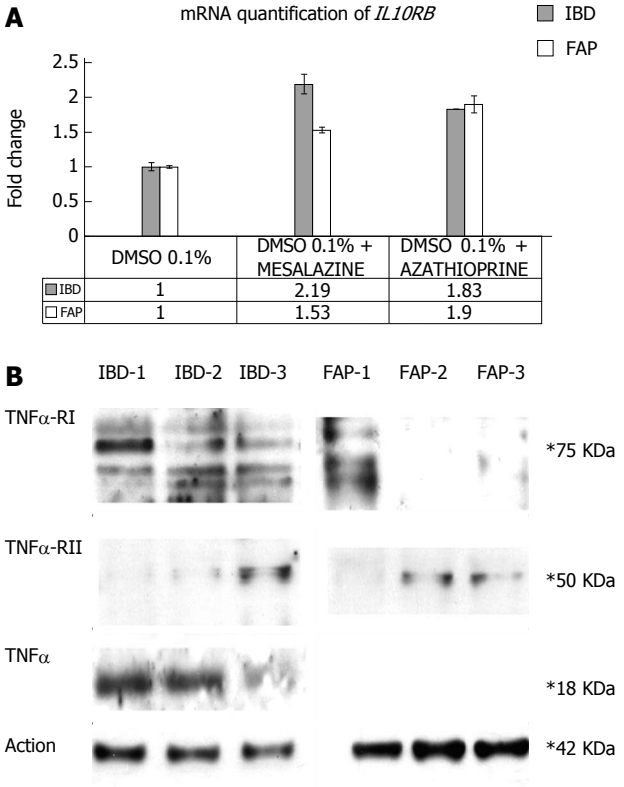

Figure 4 Effects of mesalazine and azathioprine on inflammatory bowel diseases and familial adenomatous polyposis primary fibroblasts. A: Real time polymerase chain reaction (PCR) quantification analysis of interleukin-10 (*IL10*) mRNA; Real time RT-PCR quantification analysis was performed for *IL10RB* mRNA on primary fibroblasts extracted from an inflammatory bowel diseases (IBD) and a familial adenomatous polyposis (FAP) patient and incubated with mesalazine and azathioprine; B: Western blotting assay of tumour necrosis factor α receptors-I (TNFRI) and TNFRII and tumour necrosis factor α (TNFα) performed on protein extracts from primary fibroblasts of an IBD and of a FAP patient. IBD-1: Protein extract of the IBD proband primary fibroblasts incubated with 0.1% DMSO only; IBD-2: Protein extract of the IBD proband primary fibroblasts incubated with 0.1% DMSO and mesalazine; IBD-3: Protein extract of the IBD proband primary fibroblasts incubated with 0.1% DMSO and azathioprine; FAP-1: Protein extract of the FAP patient's primary fibroblasts incubated with 0.1% DMSO only; FAP-2: Protein extract of the FAP patient's primary fibroblasts incubated with 0.1% DMSO and mesalazine; FAP-3: Protein extract of the FAP patient's primary fibroblasts incubated with 0.1% DMSO and azathioprine.

risk may reside in a regulatory region (*e.g.*, promoter) in linkage disequilibrium with these SNPs and suggest that this risk haplotype exerts a mild phenotype in the general population resulting in disease only in the presence of other genetic variants or environmental triggers[16].

As suggested by Moran *et al*[16] and also described for other human diseases[22], our results confirm that early-onset IBD could be attributed to a synergistic effect of several variant alleles of the genes encoding *IL10* receptors. These variants, alone, could only give rise to a sub-clinical manifestation of the disease. In fact, the proband's father and his brother, both carriers of homozygous A/A polymorphism E47K for the *IL10RB* gene but without the -413G->T promoter mutation in the *IL-10RA* gene, were apparently not affected. The proband's mother shows a genotype very similar to the proband. In fact, they are both heterozygous for the E47K *IL10RB* gene polymorphism and for the -413G->T promoter mutation in the *IL10RA* gene. They show different mRNA expression for the *IL10RA* gene and quantitative real-time PCR revealed a 0.1 and 0.6-fold change for the *IL10RA* mRNA in the proband and his mother, respectively. This different gene expression could be due to other intragenic SNPs in the *IL10RA* gene whose alleles are different, such as, the rs.:2256111, localised in exon 4 (c.549A->G; p.153Ala->Ala), the rs.:2229113, localised in exon 7 (c.1051A->G; p.351Arg->Gly) and the rs.:9610, localised in the 3'UTR (c.2543G->A), that were homozygous G/G, G/G and A/A in the proband but A/G heterozygous in all other family members. However, we cannot rule out other gene expression regulatory mechanisms. Possibly due to the different *IL10RA* mRNA expression, the proband's mother has not developed the disease. However, she referred to an episode of rectal bleeding and shows increased levels of *COX2* mRNA expression in peripheral blood cells.

In a recent study, 66 early onset IBD patients were analysed. The authors identified 16 patients with loss-of-function mutations in the *IL10* or *IL10R* genes. A variety of mutations were discovered. Most patients were born from consanguineous parents and they carried homozygous biallelic mutations (point mutations or deletions). However, some patients also presented compound heterozygous mutations. Genotype/phenotype correlations were not clearly observed. In fact, siblings sharing the same homozygous *IL10RB* mutation showed a remarkably distinct level of disease severity, suggesting that the phenotypic manifestation is dependent on other intrinsic or extrinsic factors that remain presently unknown[21,23].

Non-coding single nucleotide polymorphisms (SNPs) can be associated with qualitative and quantitative changes. Furthermore, genetic changes may affect transcription-factor-binding sequences, locus accessibility, translational efficiency and trans-regulators such as noncoding RNAs and microRNAs[12]. Cis- or trans-expression quantitative trait loci are detected for approximately half of the IBD risk regions, indicating that allele-specific gene-expression changes contribute to disease risk[24].

Unexpectedly, we observed β-catenin and TNFRI protein over-expression in the peripheral blood cells of the proband's apparently healthy relatives more than in the proband himself. FAP and PHTS patients, but not healthy subjects, also expressed this protein, as previously described[19]. Therefore, we suggest that these proteins could represent a good candidate for molecular markers of sub-clinical disease in relatives of patients with UC. Previous studies showed that faecal calprotectin concentration in patients with CD and relatives differed significantly from controls, suggesting that there is a high prevalence of subclinical disease in first-degree relatives of these patients. This result conforms to an additive inheritance pattern in which the genetic basis for this abnormality may represent a risk factor for CD and UC[25,26].

Because no therapeutic approach was successful in patients who are carriers of IL10 pathway alterations, we investigated the effect of mesalazine and azathioprine on the expression of IL10 receptors, TNFα and TNFα receptors. In agreement with our hypothesis, we found TNFRI under-expression and TNFRII and *IL10RB* over-expression in primary fibroblasts incubated with mesalazine and azathioprine, in both the UC and FAP patients. In the UC patient only, azathioprine, but not mesalazine, induces a TNFα decrease.

These observations could suggest that these drugs are only able to partially restore IL10 pathway function in UC, by activation of *IL10RB*, but not *IL10RA*, transcription. On the other hand, under-expression of TNFRI and over-expression of TNFRII could increase the risk of colorectal cancer-associated colitis in UC patients. As described by Chang *et al*[27], TNFRI has tumour suppressor activity in the context of colitis-associated cancer, and the role of TNFRII in cell proliferation is well known.

Current therapeutic strategies for paediatric IBD include the use of exclusive enteral nutrition, corticosteroids, mesalamine, sulfasalazine, immunomodulators (azathioprine, 6-mercaptopurine, methotrexate) and anti-TNFα-antibodies[22,28]. Aminosalicylates are the undisputed first-line option for treating and maintaining remission in UC[29]. However, the role that these drugs may play in the management of Crohn's disease has been controversial. Thiopurine drugs, azathioprine and mercaptopurine, have been shown to be effective in inducing and maintaining remission in IBD[30]. Most epidemiological studies have shown that the chronic use of 5-ASA in IBD has chemopreventive effects on the development of CRC[14,31], although some studies failed to show this, as described by Velayos *et al*[32].

TNF signals *via* two cell surface receptors, TNFRI and TNFRII, resulted in several, sometimes opposing, cellular responses that vary by context and cell nature[33,34]. In the colonic mucosa, TNF is involved in both cell survival and cell death[35]. Additionally, increased levels of TNF have been found in the setting of cancers, including those of the pancreas, skin, and ovaries[36]. With specific regard to colon carcinogenesis, TNF activity has been shown both to promote and to protect from neoplastic transformation[37-39] and there are case studies of development of cancer in other organ systems (lymphatic and skin) following the use of anti-TNF for IBD or rheumatological disease[40]. For this reason, we investigated protein expression of TNF receptors in colon mucosa of the UC patient compared to that of normal and cancer colon mucosa from patients affected by FAP and sporadic colorectal cancer. In agreement with the hypothesis suggested by Chang *et al*[27] about the tumour suppressor activity of TNFRI in the context of colitis-associated carcinogenesis, we found not only a decrease in the expression of TNFRI but also of TNFRII in colorectal cancer when compared to normal colon mucosa for each patient. The expression of TNF receptor proteins in colon mucosa of our UC patient was at an intermediate level between that observed in colorectal tumour tissue and normal mucosa of CRC patients.

In conclusion, our results, in agreement with data from recently published literature[5,16,22], indicate that early-onset UC could be caused by a synergistic effect of more variant alleles of the *IL10* receptors gene, resulting in alteration of the IL10 pathway. In our opinion, a dosage model of nonallelic non-complementation fits well with this case, whereby mutations in two different genes can behave as alleles of the same locus by causing or exacerbating the same phenotype. However, we cannot exclude, as described for others syndromes, that different mechanisms, such as alternative splicing mechanisms[41,42] or allelic variants of modifier genes, could contribute to the observed phenotypic variability[22].

In addition, we suggest that the expression of β-catenin and TNFRI protein could represent molecular markers of sub-clinical disease in apparently healthy relatives of patients. Recent findings suggest that chronic inflammation in IL10-/- mice increased P-β-catenin552 expression. Moreover, TNFRI exerts its tumour suppressor activity by modulating activation of β-catenin and controlling epithelial proliferation[43]. It clearly appears that classical therapeutic approaches do not seem adequate for IBD patients who are carriers of IL10 pathway alterations because under-expression of TNFRI signalling would confer increased risk of developing colitis associated-carcinoma. Allogenic hematopoietic stem cell transplantation could represent a causal therapeutic approach for IL10R-deficient patients, useful for the treatment of the intractable ulcerating enterocolitis of the infant, as recently suggested[14,15,20-22].

COMMENTS

Background

Inflammatory bowel diseases (IBD) are chronic relapsing inflammatory disorders thought to result from an inappropriate and continuing inflammatory response to commensal microbes in a genetically susceptible host. Mutations in interleukin-10 (*IL10*) or its receptor lead to a loss of IL10 function and cause severe intractable enterocolitis in infants and small children.

Research frontiers

Increased numbers and activation of immune cells in the intestinal mucosa enhance local levels of tumour necrosis factor-α (TNFα) and several proinflammatory IL. Recent work has demonstrated that IBD with an early onset can be monogenic and *IL10* polymorphisms have been associated with IBD in genome-wide association studies. The aims of this work were to clarify the molecular basis of disease in this young child, shedding light on a synergistic effect of *IL10RA* and *IL10RB* polymorphisms. The authors also assessed the possible presence and inheritance of subclinical intestinal inflammation in apparently healthy relatives of this patient with ulcerative colitis (UC).

Innovations and breakthroughs

Recent studies have shown that loss-of-function mutations in *IL10RA*, *IL10RB* and *IL10* genes, in immunodeficient patients, are associated with severe, infantile-onset IBD. In particular, literature reports have highlighted the role of *IL10RA* polymorphisms in the risk for developing very early onset UC. This is the first study reporting that *IL10RA* polymorphisms could have synergistic effect with those of *IL10RB*. The authors propose that these risk polymorphisms exert a mild phenotype in the general population resulting in disease only in the presence of other genetic variants in the *IL10RA* or *IL10RB*. Furthermore, these observations would suggest an inherited abnormality of beta catenin and TNFRI in the proband's relatives.

Applications

This work expands the understanding of the complex inheritance pattern of very early onset ulcerative colitis. It seems possible that the subclinical phenotypic manifestations identified in the first-degree relatives of the proband represents the consequence of inherited defects of *IL10R* genes, which then represent one of the risk factors for the disease. This study could contribute to identifying at-risk families for very early onset UC allowing clinicians to perform genetic tests and appropriate care.

Terminology

IL10 is an anti-inflammatory cytokine secreted by a variety of cell types and is critical for maintaining immune homeostasis in the gastrointestinal tract. IL10 activates downstream signalling by binding to IL10R, comprised of two α subunits (encoded by *IL10RA*) and two beta subunits (encoded by *IL10RB*).

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The authors investigated the molecular cause of very early-onset inflammatory bowel disease in an 18-mo-old child as well as his relatives. They concluded that a synergistic effect of several variant alleles of the *IL10 receptor* genes, inherited in a Mendelian manner, is involved in IBD onset in this young child. This study supports a special enthusiasm about the potential power of genomics to define the aetiology and/or phenotype of diseases. When a single specific case or family is studied, the discovery of new functional polymorphisms and the functional consequences of these mutations deserves attention even if the functional characterisation and the real pathogenic contribution of susceptible genes are hard to assess in complex disorders such as IBD.

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BRIEF ARTICLE

Dietary-suppression of hepatic lipogenic enzyme expression in intact male transgenic mice

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Abstract

AIM: To study, in intact male transgenic mice, the effects of three diets based on olive oil and olive oil diet supplemented with lovastatin and orlistat on hepatic lipogenic enzymes expression, considered markers of cell proliferation.

METHODS: Forty $Apc^{Min/+}$ mice were randomly divided into 4 groups and fed for 10 wk: olive oil (OO) group, *n* = 10 animals received a diet with olive oil 12%; olive oil plus lovastatin (LOVA) group, *n* = 10 animals received the same diet with olive oil supplemented with lovastatin 5 mg/kg; olive oil plus orlistat (OR) group, *n* = 10 animals fed the diet with olive oil supplemented with orlistat 50 mg/kg and SD group, *n* = 10 animals fed a standard diet. The activity of lipogenic enzymes and their gene expression were evaluated by radiometric and real-time reverse transcription-polymerase chain reaction assay, respectively.

RESULTS: After 10 wk of dietary treatment, the body weight was no different among animal groups (21.3 ± 3.1 g for standard group, 22.1 ± 3.6 g for OO group, 22.0 ± 3.2 g for LOVA group and 20.7 ± 3.4 g for OR group, data expressed as mean ± SD), observing a generalized well-being in all animals. All the dietary managed treated groups presented significantly reduced hepatic levels of fatty acid synthase, farnesyl pyrophosphate synthase and 3-hydroxyl-3-methyl-glutaryl CoA reductase activity and gene expression when compared with the mice fed the standard diet. To evaluate cell proliferation in the liver of treated mice, the levels of cyclin E mRNA have been measured, demonstrating a significant reduction of *cyclin E* gene expression in all treated groups. Evidence of reduced hepatic cell proliferation was present overall in OO group mice.

CONCLUSION: We confirm the role of lipogenic enzymes as markers of cell proliferation, suggesting that appropriate dietary management alone or with drugs can be a feasible approach to counteract hepatic cell proliferation in mice.

Key words: Lipogenic enzymes; Liver; Markers of cell proliferation; Transgenic mice; Dietary treatment

Core tip: The olive oil diet significantly reduces the enzymatic activities, as well as the expression of hepatic cell cycle related genes. The addition of drugs as lovastatin and orlistat to olive oil diet more down-regulated the studied lipogenic enzymes, demonstrating that the inhibition of these enzymes with natural components of diet could have a potential benefit in association with canonical chemical substances to counteract hepatic

cell proliferation in mice.

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INTRODUCTION

Several alterations of lipid metabolism are often found in tumors, where neoplastic lipogenesis is essential for cancer cell survival[1]. Cancer cells esterify fatty acids predominantly to phospholipids, essential component of cell membranes. The main pathway through which proliferating cells gain lipids for membrane synthesis is the endogenous mevalonate pathway[2,3]. Increased synthesis of mevalonate and mevalonate derived isoprenoids supports increased cell proliferation through the activation of growth-regulatory proteins and oncoproteins and by promoting DNA synthesis[4,5].

Endogenous fatty acid synthesis is dependent on the activity of fatty acid synthase (FAS). This enzyme is over-expressed in many types of malignancies, including prostate, breast, lung and colon cancer[6-8]. The tumor environment contains regions of poor oxygenation and high acidity, and FAS over-expression could confer a selective growth advantage upon these unfavorable conditions[9].

It is known that hydroxyl-3-methyl-glutaryl CoA reductase (HMGCoAR) activity is up-regulated severalfold in colon tumors and not regulated by feedback inhibition from cholesterol compared with normal mucosa[2,10,11]. Alterations in biosynthetic processes of the mevalonate pathway and in the levels of enzyme products participating in this biochemical system may contribute to the cell growth advantage acquired during carcinogenic process and to the development of malignancy.

In cancer, high levels of mevalonate-derived metabolites, such as isoprenoid compounds have been demonstrated[5,12,13]. Several HMGCoA metabolites, such as farnesyl pyrophosphate (FPP) and geranyl pyrophosphate are implicated in oncogene activation and tumorigenesis[14]. FPP, produced by activity of FPP synthase, is the substrate for the farnesylation of a wide number of proteins implicated as potential growth regulators. FPP synthase gene is over-expressed in different human tumors as well as an upregulation of FPP synthase has been detected in about 85% of hepatocellular carcinoma[15]. An elevated FPP synthase expression has also been observed in rat prostate tumor cell lines[16].

Previously, we demonstrated an high FPP synthase activity in human colorectal cancer[17].

The regulation of lipogenic enzymes abundance in cancer cells is complex and occurs at the transcriptional or posttranscriptional levels. Several studies show that blockade of these enzymes can attenuate the growth and survival of tumor cells[1,18,19], being potential target for cancer therapy.

Moreover, promotion and progression of carcinogenesis are susceptible to nutritional interventions aimed at counteracting cancer development[20]. In this respect, olive oil consumption has been demonstrated to reduce the incidence of aberrant crypt foci in azoxymethane-treated rats[21]. Furthermore, olive oil is able to down-regulate the expression of cyclooxygenase-2 and BCL-2 proteins that plays a crucial role in colorectal carcinogenesis[22]. Olive oil healthy effects can be attributed not only to the higher relationship between unsaturated and saturated fatty acids, but also to the antioxidant property of its phenolic compounds, as oleuropein and hydroxytyrosol (HT). As antioxidants, polyphenols may protect cell constituents against oxidative damage and act as highly effective chemopreventive agents[23,24].

Olive oil polyphenols are quickly absorbed by intestine, but the biotransformation of absorbed HT should take place mostly in the liver. Taking into account this point, the aim of the present study was to test in the $Apc^{Min/+}$ mouse model three diets based on olive oil and olive oil diet supplemented with lovastatin and orlistat, known agents with antitumor activity and inhibitors of HMGCoAR and FAS, respectively. Since high serum concentration of the lipid and liver steatosis have been observed in $Apc^{Min/+}$ mice[25,26], this experimental model was selected to evaluate a putative hepatic dietary-induced down-regulation of lipogenic enzymes.

MATERIALS AND METHODS

Animals and experimental study design

Five-week-old C57BL/6J mice with an heterozygote mutation for the Apc gene ($Apc^{Min/+}$) were obtained from Charles River (Calco, CO, Italy). The mice were maintained in the animal care facility at our Institute. They were kept in temperature, air- and light-controlled conditions and received food and water *ad libitum*. Animals did not receive any surgical or hormonal manipulation but were kept anatomically and physiologically intact. All animals received care in compliance to the "Guide for the Care and Use of Laboratory Animals". The procedures related to animal use have been communicated to the Italian Ministry of Health and approved.

Forty $Apc^{Min/+}$ mice were randomly divided into 4 groups and fed for 10 wk: olive oil (OO) group, n = 10 animals received a diet with olive oil 12% (12.5% protein, 12% oils and fats, 3% fibers); lovastatin (LOVA) group, n = 10 animals received the same diet with olive oil supplemented with lovastatin 5 mg/kg; orlistat (OR) group, n = 10 animals fed the diet with olive oil supplemented with orlistat 50 mg/kg and SD group, n = 10 animals fed a standard diet (18.5 % protein, 5% oils and fats, 4.2% fibers). Any diet was provided in pellets by Mucedola Srl, Settimo Milanese, Italy. Body weight and food intake were measured every 3 d.

After 10 wk of dietary treatment, the animals were killed by cervical dislocation. The liver from each animal was immediately excised and washed with phosphate buffered saline. Samples of fresh liver tissue were rapidly frozen and stored at -80 ℃ and the counterpart specimens were fixed in 10% buffered formalin to assess histological analysis.

FAS activity assay

FAS activity was determined on frozen liver samples. After tissue homogenization and centrifugation, an aliquot of supernatant (50 μL) was pre-incubated with 100 mmol/L potassium phosphate buffer, pH = 7 for 15 min at 37 ℃. Subsequently, 20 μL of reaction mix (2.5 mmol/L NADPH, 1.25 mmol/L acetyl-CoA, 1.25 mmol/L malonyl-CoA and 0.02 mmol/L 2-^{14}C-malonyl-CoA (52 mCi/mmol, Amersham Biosciences, United Kingdom) were added and samples were incubated for 10 min at 37 ℃. Reactions were stopped by the addition of 500 μL 1 mol/L HCl/methanol (6:4, v:v); fatty acids were extracted with 1 mL of petroleum ether and incorporation of 2-^{14}C-malonyl-CoA was analyzed by scintillation counting. FAS activity was expressed as picomoles of incorporated 2-^{14}C-malonyl-CoA per minute per milligram of total proteins (pmol/min/mg prot).

Preparation of microsomal fraction

Frozen hepatic tissue specimens were placed in cold homogenization buffer containing 0.3 mol/L sucrose, 10 mmol/L EDTA (pH = 7.4) and 1 mmol/L 2-β-mercaptoethanol. Each homogenate was centrifuged at 900 × *g* for 5 min at 4 ℃; the supernatant was further centrifuged at 8700 *g* for 10 min; the pellet was discarded and the supernatant was centrifuged at 10000 *g* for 10 min to obtain microsomal fraction. Each pellet was resuspended in 0.2 mL ice-cold buffer containing 20 mmol/L imidazol (pH = 7.4), 5 mmol/L dithiothreitol.

FPP synthase activity assay

FPP synthase assay was carried out with some modifications of the procedure of Krisans *et al*[13] and described by Gupta *et al*[27]. Briefly, FPP synthase was assayed in 150 μL containing 25 mmol/L Hepes, pH = 7, 2 mmol/L $MgCl_2$, 1 mmol/L dithiothreitol, 5 mmol/L KF, 1% noctyl-β-glucopyranoside, 3.3 μmol/L [4-14C] IPP (18 Ci/mmol), 3 μmol/L unlabeled IPP and 20 μmol/L geranyl diphosphate. Reactions were started by adding 40 μL of microsomal fraction containing 100 μg of total protein and incubated for 45 min at 37 ℃. Reactions were stopped by the addition of 150 μL 2.5 mol/L HCl in 80% ethanol containing 100 μg/mL farnesol as a carrier. The samples were hydrolyzed for 30 min at 37 ℃ to convert the FPP to farnesol and neutralized by the addition of 150 μL of 10% NaOH. The reaction product (farnesol) was extracted into 1 mL of *N*-hexane and an aliquot (200 μL) of the organic phase was used for radioactivity counting. One unit of enzyme activity is defined as the amount of enzyme required to synthesize one pmol of FPP per min. Parallel samples were assayed to evaluate the total and the nonspecific radioactivity. In all experiments, enzyme assays were carried out in duplicate. The coefficient percentages of intra- and inter-assay variation were 3% and 4%, respectively.

Table 1 Sequences of amplification primers

Gene		Primer
FPP synthase	Sense	5′-AAAATTGGCACTGACATCCAGG-3′
	Antisense	5′-GGGTGCTGCGTACTGTTCAATG-3′
FAS	Sense	5′-GATCCTGGAACGAGAACACGA-3′
	Antisense	5′-GAGACGTGTCACTCCTGGACTTG-3′
HMGCoAR	Sense	5′-GCTTGAGCATCCTGACATAC-3′
	Antisense	5′-GAACCATAGTTCCCACGTCT-3′
Cyclin E	Sense	5′-GTCTTCGCAGATCGCAGA-3′
	Antisense	5′-GAGACCTTCTGCGACTCCA-3′
β-actin	Sense	5′-GCCTCTGGTCGTACCACTGGC-3′
	Antisense	5′-AGGGAGGAAGAGGATGCGGCA-3′

FPP: Farnesyl pyrophosphate; FAS: Fatty acid synthase; HMGCoAR: 3-hydroxyl-3-methyl-glutaryl CoA reductase.

HMGCoAR activity assay

HMGCoAR activity was measured by radiochemical assay using *DL*-3-hydroxy-3-methyl-[3-^{14}C]-glutaryl-coenzyme A (^{14}C-HMGCoA) as substrate. Briefly, 50 μL of microsomal fraction containing about 50 μg of total protein were pre-incubation for 10 min at 37 ℃ with 50 μL of cofactors solution containing 1 mol/L potassium-phosphate buffer (pH = 7.4), 100 mmol/L EDTA (pH = 7.4), 50 mmol/L dithiothreitol, 200 mmol/L glucose-6-phosphate, 25 mmol/L NADP and 0.5 U of glucose-6-phosphate dehydrogenase. Reactions were started by adding 10 μL of ^{14}C-HMGCoA (specific activity 5.0 mCi/mmol) in each sample followed by an incubation for 20 min at 37 ℃. Reactions were stopped by the addition of 20 μL of KOH 33% and incubated for 45 min to consent the hydrolysis of substrate. The subsequent addition of 50 μL of 5 mol/L HCl and the incubation for 60 min at 37 ℃ were performed to convert the formed [^{14}C]-mevalonic acid in [^{14}C]-mevalonolactone. The reaction product (^{14}C-mevalonolactone) was extracted into 300 μL of *N*-hexane and an aliquot (200 μL) of the organic phase was used for radioactivity counting. HMGCoAR activity was expressed as picomoles of [^{14}C]-mevalonate formed per minute per milligram of microsomal proteins (pmol/min/mg prot). In all experiments, enzyme assays were carried out in duplicate.

Gene expression analysis

Total RNA from samples of liver tissue was isolated with TRI-Reagent (Mol. Res. Centre Inc. Cincinnati, O, United States), following the manufacturer's instruction. Briefly, the tissue was homogenized in 0.25 mL of cold 0.9% NaCl; then, 0.75 mL of TRI-Reagent and 0.2 mL of chloroform were added to the homogenate. The samples were vigorously shaken and centrifuged and the RNA present in the aqueous phase was precipitated with 0.5 mL of isopropanol. The RNA pellet was washed

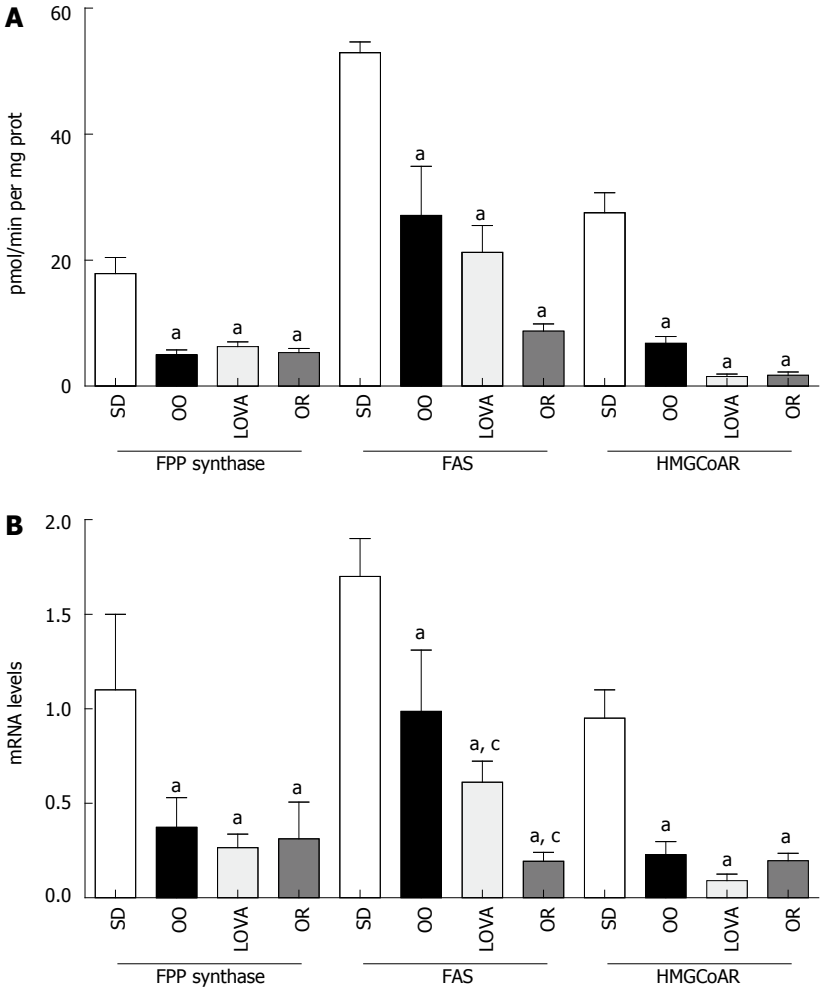

Figure 1 Farnesyl pyrophosphate synthase, fatty acid synthase and 3-hydroxyl-3-methyl-glutaryl CoA reductase activity, mRNA levels in the four groups of treatment. A: Activity levels in the four groups of treatment. [a]$P < 0.05$ *vs* SD group (one-way analysis of variance and Tukey's Multiple Comparison Test). Data are expressed as mean ± SE; B: mRNA levels in the four groups of treatment. [a]$P < 0.05$ *vs* SD group (one-way analysis of variance and Tukey's Multiple Comparison Test); [c]$P < 0.05$ *vs* OO group (Tukey's Multiple Comparison Test). Data expressed as *n* molecules mRNA target gene/*n* molecules mRNA β-actin. SD: Standard diet; OO: Olive oil; LOVA: Olive oil plus lovastatin; OR: Olive oil plus orlistat; FPP: Farnesyl pyrophosphate; FAS: Fatty acid synthase; HMGCoAR: 3-hydroxyl-3-methyl-glutaryl CoA reductase.

once with 1 mL of 75% ethanol, dried, resuspended in sterile water and quantified by UV absorbance. 2 μg of total RNA were used for the reverse transcription reaction performed in 20 μL of final volume at 41 ℃ for 60 min, using 30 pmol of antisense primer (Table 1) for analyses of the FAS, FPP synthase, HMGCoAR, cyclin E and the β-actin gene. β-actin gene was utilized as reference gene. Real-time PCRs were performed in 25 μL of final volume containing 2 μL of cDNA, master mix with SYBR Green (iQ SYBR Green Supermix Bio-Rad, Milan, Italy) and sense and antisense primers for the *FAS*, *FPP* synthase, *HMGCoAR*, *cyclin E* and the *β-actin* gene (Table 1).

Real-time polymerase chain reaction (PCR) was carried out in an CFX96 Real-time PCR Detection System (Bio-Rad Laboratories, Inc.) using the following protocol: 45 cycles at 95 ℃ for 3 min, 95 ℃ for 10 s, 55 ℃ for 30 s followed by a melting curve step at 65 ℃-95 ℃ with a heating rate of 0.5 ℃ per cycle for 80 cycles. The PCR products were quantified by external calibration curves, one for each tested gene, obtained with serial dilutions of known copy number of molecules (10^2-10^7 molecules). All expression data were normalized by dividing the target amount by the amount of β-actin used as internal control for each sample. The specificity of the PCR product was confirmed by gel electrophoresis.

Statistical analysis

The significance of the differences among experimental groups was evaluated with one-way analysis of variance (ANOVA) and Tukey's Multiple Comparison Test. Differences were considered significant at a 5% probability level.

RESULTS

After 10 wk of dietary treatment, the body weight (in grams) was no different among animal groups (21.3 ± 3.1 g for standard group, 22.1 ± 3.6 g for OO group, 22.0 ± 3.2 g for LOVA group and 20.7 ± 3.4 g for OR group, data expressed as mean ± SD), observing a generalized well-being in all animals.

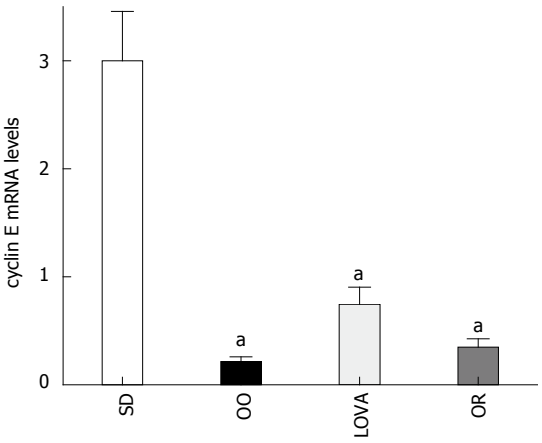

Figure 2 Cyclin E mRNA levels (mean ± SE) in the four groups of treatment. Data expressed as *n* molecules mRNA cyclin E gene/*n* molecules mRNA β-actin. $^{a}P < 0.05$ *vs* SD group (one-way analysis of variance and Tukey's Multiple Comparison Test). SD: Standard diet; OO: Olive oil; LOVA: Olive oil plus lovastatin; OR: Olive oil plus orlistat.

All the dietary managed treated groups presented significantly reduced levels of hepatic lipogenic enzymes activity when compared with the mice fed the standard diet, being such reduction particularly marked in LOVA group for HMGCoAR and in OR group for FAS (one-way analysis of variance and Tukey's Multiple Comparison Test, $P < 0.05$) (Figure 1A).

The levels of FPP synthase, FAS and HMGCoAR mRNA in liver tissue followed the same behavior of protein activities (Figure 1B). Differences statistically significant were observed between the three groups of treatment and the mice group treated with standard diet for all enzymes studied (one-way analysis of variance and Tukey's Multiple Comparison Test, $P < 0.05$). For FAS mRNA levels, a significant reduction was also detected between LOVA group and OR group compared to OO group (Figure 1B).

To evaluate cell proliferation in the liver of treated mice, the levels of cyclin E mRNA have been measured, demonstrating a significant reduction of *cyclin E* gene expression in all treated groups. Evidence of reduced hepatic cell proliferation was present overall in OO group mice (Figure 2).

DISCUSSION

Our data support appropriate dietary management as a feasible approach to counteract hepatic cell proliferation in mice, targeted to the selective down regulation of FAS, FPP synthase and HMGCoAR considered biomarkers of cell proliferation.

Understanding the distribution of roles within a biochemical pathway is clearly important and it provides a rationale for selecting a particular reaction step suitable for therapeutic intervention.

Statins having biochemical effects on cholesterol synthesis, are considered as potential anti-tumor agents[28], inhibiting tumor cell growth by restricting either cholesterol availability or cholesterol synthesis[28,29]. Previously, we have demonstrated that the combined treatment with eicosapentaenoic acid (EPA) and lovastatin enhanced the regulatory effect on gene expression of HMGCoAR and low density lipoprotein receptor in HepG2 cell line[30]. Moreover, we detected a synergistic effect in the inhibition of cancer cell proliferation obtained by combination of EPA and Lovastatin, demonstrating an inhibition at the lower doses with respect to the substances used separately.

On the other hand, orlistat, as an anti-obesity drug, is a novel and selective FAS inhibitor in tumors[1]. Moreover, FAS inhibition by orlistat reduces proliferation and promotes apoptosis in prostate, breast and gastric cancer cell lines[31,32].

In our previous study[33], we showed a down-regulation of FAS observed after HT treatment in SW620 cell line, suggesting that FAS might be a molecular target for anti-proliferative activity of olive oil polyphenols in a metabolically defined subset of colon cancer.

In this study the reduction of *cyclin E* gene expression in mice liver by these compounds demonstrates their ability to inhibit cell proliferation. This finding supports the role of lipogenic enzymes as markers of cell growth.

The olive oil diet significantly reduces the enzymatic activities, as well as the expression of hepatic cell cycle related genes. The addition of drugs as lovastatin and orlistat to olive oil diet more down-regulated the studied lipogenic enzymes, demonstrating that the inhibition of these enzymes with natural components of diet could have a potential benefit in association with canonical chemical substances to counteract hepatic cell proliferation in mice.

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COMMENTS

Background

Several alterations of lipid metabolism are often found in tumors, where neoplastic lipogenesis is essential for cancer cell survival. Increased synthesis of mevalonate and mevalonate derived isoprenoids supports increased cell proliferation through the activation of growth-regulatory proteins and oncoproteins and by promoting DNA synthesis. Promotion and progression of carcinogenesis are susceptible to nutritional interventions aimed at counteracting cancer development. In this respect, olive oil consumption has been demonstrated to reduce the incidence of colorectal cancer. Olive oil healthy effects can be attributed not only to the higher relationship between unsaturated and saturated fatty acids, but also to the antioxidant property of its phenolic compounds, as oleuropein and hydroxytyrosol.

Research frontiers

Alterations in biosynthetic processes of the mevalonate pathway and in the levels of enzyme products participating in this biochemical system may contribute to the cell growth advantage acquired during carcinogenic process and to the development of malignancy. The blockade of these enzymes can attenuate the growth and survival of tumor cells, being potential target for cancer therapy. Their paper demonstrates that the inhibition of lipogenic enzymes with natural components of diet could have a potential benefit in association with canonical chemical substances to counteract hepatic cell proliferation in mice.

Innovations and breakthroughs

The study supports the role of lipogenic enzymes as markers of cell growth. Reported data on possible benefits of olive oil to counteract hepatic cell proliferation in mice, highlight the importance and the innovation that an appropriate dietary treatment can be useful in cancer prevention.

Applications

Further studies will be designed to translate the findings in clinical practice. Olive oil through its ability to suppress the lipogenic enzymes may provide well-tolerated novel therapies, particularly in metabolic disorders-related tumors, as gastrointestinal cancers.

Terminology

Lipogenic enzymes are involved in lipid metabolism. The lipogenesis is essential for cell membrane synthesis. The main pathway through which proliferating cells gain lipids for membrane synthesis is the endogenous mevalonate pathway, where the 3-hydroxyl-3-methyl-glutaryl CoA reductase (HMGCoAR) is the key enzyme. Among HMGCoAR metabolites, farnesyl pyrophosphate (FPP), produced by activity of FPP synthase, is the substrate for the farnesylation of a wide number of proteins considered potential growth regulators. Moreover, endogenous fatty acid synthesis is dependent on the activity of fatty acid synthase (FAS). FAS is a multi-enzyme protein containing domains for acyl-carrier peptide and the seven different catalytic activities required for the conversion of acetyl-CoA and malonyl-CoA to palmitate. Expression of FAS is linked to specific functions such as the conversion and storage of energy in the liver and in adipose tissue, and is possibly involved in the regulation of food intake.

Peer review

The aim of this research is to study the effects of three diets based on olive oil and olive oil diet supplemented with lovastatin and orlistat on hepatic lipogenic enzymes expression in $Apc^{Min/+}$ mice which are divided randomly into 4 groups of 10 animals per group that were fed for 10 wk: Concentration of olive oil used was 12%; lovastatin 5 mg/kg; orlistat 50 mg/kg and SD group that was fed a standard diet. The activity of lipogenic enzymes and their gene expression were evaluated by radiometric and real-time reverse transcription-polymerase chain reaction assay. Results show that all the dietary managed treated groups significantly reduced hepatic levels of fatty acid synthase, farnesyl pyrophosphate synthase and 3-hydroxyl-3-methyl-glutaryl CoA reductase activity and gene expression when compared with the mice fed the standard diet. The data are the potential interest and they confirm the role of lipogenic enzymes as markers of cell proliferation, suggesting that appropriate dietary management alone or with drugs can be a feasible approach to counteract hepatic cell proliferation in mice. The conclusions indicate that the observed effects could serve as markers for hepatic cell proliferation.

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BRIEF ARTICLE

Alanine aminotransferase normalization at week 8 predicts viral response during hepatitis C treatment

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Abstract

AIM: To investigate alanine aminotransferase (ALT) and sustained virological response (SVR) in chronic hepatitis C (CHC) during peginterferon-ribavirin treatment.

METHODS: One hundred and fifty-one genotype 1 CHC patients underwent treatment for 48 wk with peginterferon and ribavirin, and were retrospectively divided into two groups as having a rapid virological response (RVR) (Group 1, *n* = 52) and not having an RVR (Group 2, *n* = 99). We also subdivided each group into two according to the initial ALT level being high (Group 1h and Group 2h) or normal (Group 1n and Group 2n). HCV RNA and ALT levels were measured at baseline; at 4, 12, 24 and 48 wk during the treatment period; and at 24 wk follow-up. ALT levels were also obtained at 8 wk. According to the results of ALT, patients were enrolled in either the follow-up abnormal or follow-up normalized ALT groups at each interval. Patients with high and normal ALT levels were compared for each interval in terms of SVR.

RESULTS: The SVR rates were 83% *vs* 40% (*P* = 0.000), 82% *vs* 84% (*P* = 0.830), and 37% *vs* 44% (*P* = 0.466) when comparing Group 1 with 2, 1h with 1n, and 2h with 2n, respectively. In Group 2h, the SVR rates were 34% *vs* 40% (*P* = 0.701), 11% *vs* 52% (*P* = 0.004), 12% *vs* 50% (*P* = 0.007), 7% *vs* 50% (*P* = 0.003), 6% *vs* 53% (*P* = 0.001), and 0% *vs* 64% (*P* = 0.000) when comparing patients with high and normalized ALT levels at week 4, 8, 12, 24, 48 and 72, respectively. The multiple logistic regression analysis revealed that RVR (OR = 7.05; 95%CI: 3.1-16.05, *P* = 0.000), complete early virological response (cEVR) (OR = 17.55; 95%CI: 6.32-48.76, *P* = 0.000), normalization of ALT at 8 wk (OR = 3.04; 95%CI: 1.31-7.06, *P* = 0.008), and at 12 wk (OR = 4.21; 95%CI: 1.65-10.76, *P* = 0.002) were identified as independent significant predictive factors for SVR.

CONCLUSION: Normalization of ALT at 8 wk may predict viral response during peginterferon-ribavirin treatment in genotype-1 CHC patients especially without RVR.

Key words: Chronic hepatitis C; Genotype-1; Alanine aminotransferase; Rapid virological response; Sustained virological response, Interferon; Ribavirin

Core tip: Rapid virological response (RVR) has been acknowledged as a powerful on-treatment predictor of sustained virological response (SVR) in the treatment of chronic hepatitis C (CHC). However, RVR rates are relatively low and a new predictor is needed for CHC patients; especially those without RVR. In this context, on-treatment alanine aminotransferase (ALT) changes may be a new predictor for SVR. In this study, we found that ALT normalization at the 8 wk may be an important on-treatment predictor for CHC.

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INTRODUCTION

The sustained virological response (SVR) after combined peginterferon and ribavirin treatment in chronic hepatitis C (CHC) patients is heterogeneous[1]. Thus, for the treatment of CHC, clinicians would like to establish predictive factors for SVR[2]. Pretreatment predictive viral factors are hepatitis C virus (HCV) genotype and serum HCV RNA levels at baseline, and many host factors including age, sex, weight, race, liver fibrosis, insulin resistance and recently acknowledged presence of interleukin-28 polymorphism[1,3,4].

Once treatment is initiated, rapid virological response (RVR) is acknowledged as a powerful on-treatment predictor of SVR[5]. However, RVR rates are relatively low and a new predictor is needed for CHC patients; especially those without RVR. In this context, on-treatment alanine aminotransferase (ALT) changes may be a new predictor for SVR. There are few data evaluating the relationship between on-treatment ALT changes and SVR during combination treatment with peginterferon and ribavirin in patients with CHC.

The purpose of this study was to investigate the relationship between on-treatment ALT changes and SVR in genotype 1 CHC patients during peginterferon-ribavirin treatment.

MATERIALS AND METHODS

Patients

Medical records of patients with CHC, who were treated between 2008 and 2012 at the Adana Numune Training and Research Hospital, Turkey, were retrospectively reviewed. Eligible patients had chronic HCV genotype 1 infection with compensated liver disease and a detectable plasma HCV RNA level, and had not been previously treated for hepatitis C. Patients who were on treatment or had withdrawn because of adverse events, or were lost during follow-up were excluded from the study. Patients were also excluded if they had co-infection with hepatitis B or HIV, any other cause of liver disease such as alcohol abuse or autoimmune hepatitis, morbid obesity (Body Mass Index > 40), poorly controlled diabetes mellitus (glycated hemoglobin value > 8.5%), severe depression or a severe psychiatric disorder, or active substance abuse. Finally, 151 patients who were followed up for at least 6 mo after completion of treatment were included in the study. Most patients had undergone liver biopsy within 6 mo before screening. The liver histology was graded by the histological activity index according to the criteria of Ishak *et al*[6], which comprise two major components namely Histological Activity Index and fibrosis. The study was approved by our Institutional Review Board and was conducted in accordance with provisions of the Declaration of Helsinki and Good Clinical Practice guidelines.

Study design

We first categorized 151 patients into two main groups. Group 1 included 52 patients with RVR, and Group 2 included 99 patients without RVR. Each group was then subdivided into two according to the initial ALT level: Group 1h, patients who had initial abnormal ALT levels with RVR; Group 1n, patients who had initial normal ALT levels with RVR; Group 2h, patients who had initial abnormal ALT levels without RVR; and Group 2n, patients who had initial normal ALT levels without RVR. ALT patterns were analyzed throughout the course of treatment and follow-up period.

Treatment with peg-interferon plus ribavirin

Patients with genotype 1 infection were administered peginterferon α-2a at a dose of 180 μg/wk or peginterferon α-2b at the standard dose of 1.5 μg/kg per week; both in combination with oral ribavirin at a dose of 1000-1200 mg/d, according to body weight (< 75 kg, 1000 mg/d; ≥ 75 kg, 1200 mg/d). Patients underwent treatment for 48 wk and were followed-up for 24 wk.

Laboratory assessment

Patients were followed up by blood sample analysis and measurement of biochemical variables. Blood samples were tested for complete blood counts, serum ALT levels, HCV genotype (baseline only) and serum HCV RNA. Serum ALT levels were obtained from all patients at baseline and at weeks 4, 8, 12, 24 and 48 of combined peg-interferon and ribavirin treatment, and 24 wk after completing therapy. According to the results of ALT, patients were included in either the follow-up abnormal or follow-up normalized ALT groups at each interval. Patients with high and normal ALT levels were compared at weeks 4, 8, 12, 24, and 48 of treatment; and follow-up week 24 in terms of SVR. The upper normal limit for serum ALT was 40 IU/L in our laboratory.

Efficacy assessments

HCV RNA levels were measured with the use of the Cobas TaqMan assay (Roche Diagnostics, Milan, Italy), which has a lower limit of quantitation of 20 IU/mL. Real-time polymerase chain reaction with Rotor Gene Q (Qiagen, Milan, Italy) was used for genotype determination. Measurements were obtained at screening visits (baseline); weeks 4, 12, 24 and 48 during the treatment period; and 24 wk follow-up. The primary endpoint of efficacy was SVR (undetectable serum HCV RNA levels at 24 wk after completing treatment). RVR was defined as undetectable serum HCV RNA level at the end of 4 wk. Patients with detectable HCV RNA at week 4 (no RVR) who had undetectable HCV RNA at week 12 were said to have a complete early virological response (cEVR). End of treatment response (ETR) was defined

Table 1 Comparison of baseline characteristics and virological responses in patients with and without rapid virological response *n* (%)

	Patients with RVR (Group 1, *n* = 52)	Patients without RVR (Group 2, *n* = 99)	*P*[1] value
Age (yr)	55.9 ± 12.2	57.9 ± 11.1	0.312
Male	24 (46.2)	51 (51.5)	0.534
Initial ALT (IU/L)	87.3 ± 109.8	52.2 ± 40.4	0.005
Initial abnormal ALT level	33 (63.5)	49 (49.5)	0.103
Initial HCV RNA (log10 IU/ mL)	5.4 ± 1.1	6.1 ± 0.8	0.000
cEVR	52 (100)	58 (59)	0.000
ETR	48 (92)	56 (57)	0.000
SVR	43 (83)	40 (40)	0.000
ISHAK score, mean ± SD			
Biopsy of receipt	31 (59.6)	63 (63.6)	0.631
HAI	8.9 ± 3.8	8.2 ± 2.8	0.356
Fibrosis score	2.9 ± 1.3	2.7 ± 1.4	0.681

[1]Student's *t* test. RVR: Rapid virological response; ALT: Alanine aminotransferase; HCV: Hepatitis C virus; EVR: Early virological response; ETR: End of treatment response; SVR: Sustained virological response; HAI: Histological activity index.

as the undetectable serum HCV RNA level at the end of treatment.

Statistical analysis

Data management and statistical analyses were performed with SPSS for Windows Release 18.0.0 (SPSS Inc., Chicago, IL, United States). Results are expressed as the mean ± SD. Student's *t* test or analysis of variance was used to assess the significance of SVR rates. Univariate analysis and multiple logistic regression analysis were used to identify predictive factors for sustained response. In the multiple logistic regression analysis, we determined the strength of the influence of possible variables (RVR, cEVR, normalization of ALT at 8 and 12 wk) for sustained response. $P < 0.05$ was considered as statistically significant.

RESULTS

Characteristics and viral responses of the study patients according to RVR

RVR was achieved in 52 (34.4%) patients and cEVR was achieved in 110 (72.9%) patients. The remaining 41 patients who did not achieve a cEVR at week 12 had undetectable HCV RNA at 24 wk. Comparison of baseline characteristics and virological responses in patients with and without RVR are summarized in Table 1. The initial ALT level was higher in patients with RVR than patients without RVR, although there was no significant difference in the number of patients with initial abnormal ALT level between the groups. Initial HCV RNA (log10 IU/ mL) was significantly lower and the SVR rate was significantly higher in patients with RVR compared to patients without RVR. The overall SVR rate was 55%.

Characteristics and viral responses according to initial ALT levels in patients with and without RVR

Baseline characteristics and virological responses according to the initial ALT level in patients with and without RVR were similar (Table 2).

During treatment

Schematic diagrams showing patient group flow according to initial ALT level and subsequent pattern of changes in patients with and without RVR are shown in Figure 1.

Viral responses in patients with initial normal ALT levels during treatment

Patients who had normal initial ALT levels showed nearly sustained normal ALT levels during treatment. Only one patient in Group 1n (Figure 1A) and two patients in Group 2n (Figure 1B) had variable ALT abnormality during treatment. SVR rates were 84% and 44% in Group 1n and 2n, respectively.

Viral responses according to ALT normalization during treatment

Comparison of SVR rates in patients with high and normalized ALT levels at weeks 4, 8, 12, 24, 48 and 72 in the initial abnormal ALT level groups with and without RVR are summarized in Table 3 and illustrated in Figure 2. At 8 wk, normalization of ALT became significant in terms of SVR in both groups.

Analysis of factors that predicted SVR to combination therapy

We performed univariate analysis using the χ^2 test to investigate the association of SVR with various factors. In the multiple logistic regression for the strength of influence factors, RVR (OR = 7.05; 95%CI: 3.1-16.05, $P = 0.000$), cEVR (OR = 17.55; 95%CI: 6.32-48.76, $P = 0.000$), normalization of ALT at week 8 (OR = 3.04; 95%CI: 1.31-7.06, $P = 0.008$), and at week 12 (OR = 4.21; 95%CI: 1.65-10.76, $P = 0.002$) were identified as independent significant predictive factors for SVR.

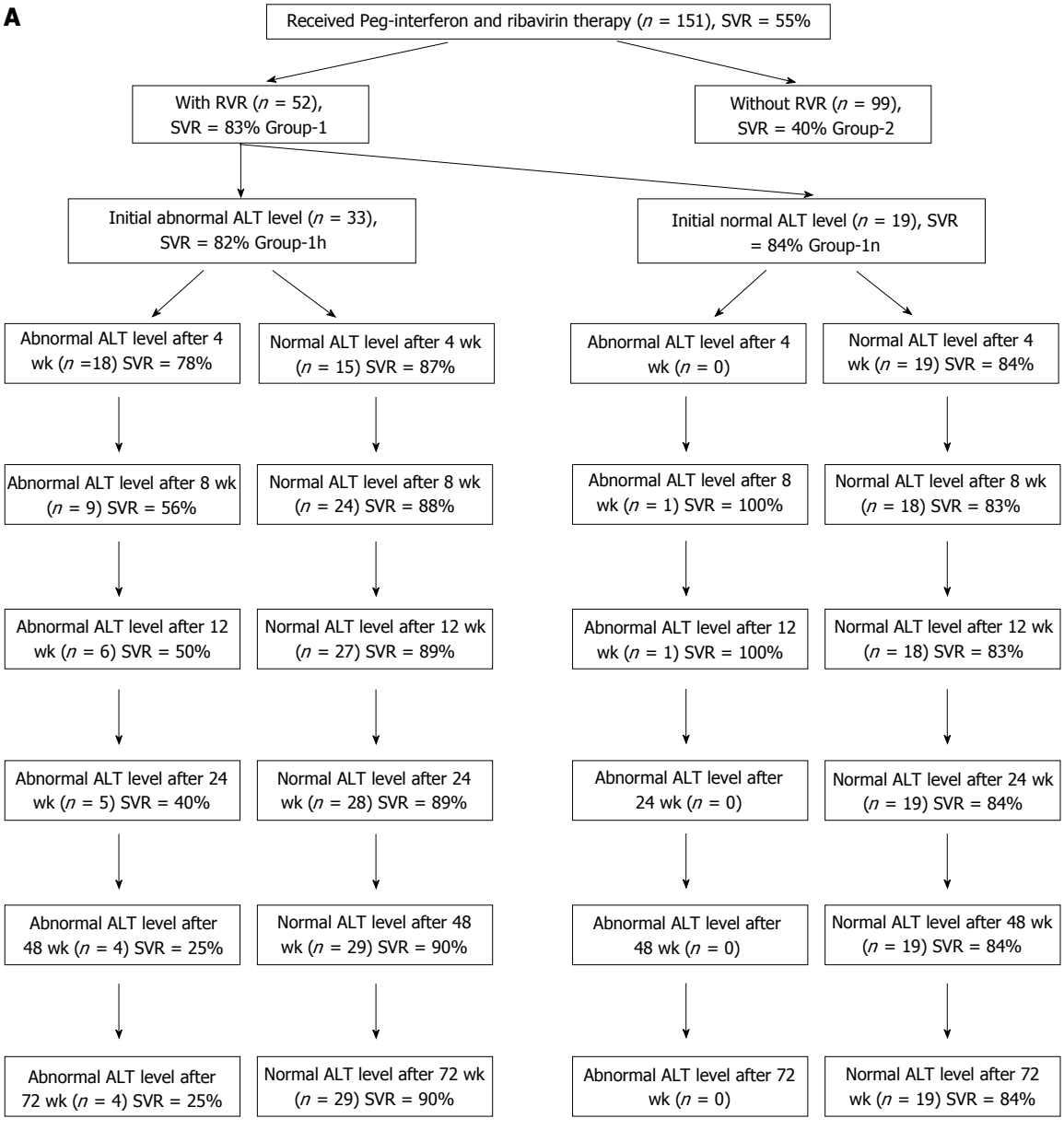
DISCUSSION

Treatment with pegylated interferon-α and ribavirin is a well-accepted standard of care for patients with CHC[2]. Although this approach appears to be highly effective for patients with HCV genotypes 2 or 3, who have a SVR of about 80%, the treatment algorithm is less effective for patients with HCV genotype 1, because these patients have SVR rates of just 40%-50%[7,8]. There are some pretreatment factors related to SVR. Clinicians need to know these factors for predicting SVR, to determine non-responders as early as possible in order to avoid prolonged treatment without benefit[2,9]. The viral factors are HCV genotype and serum HCV RNA levels at baseline and numerous host factors include age, sex, race, weight, liver fibrosis, and insulin resistance[1]. Recently, an interleukin-28 polymorphism has been acknowledged as

Table 2 Comparison of baseline characteristics and virological responses according to the initial alanine aminotransferase level in patients with and without rapid virological response *n* (%)

	Patients with RVR (Group 1)		P^1 value	Patients without RVR (Group 2)		P^1 value
	Initial abnormal ALT level (Group 1h, *n* = 33)	Initial normal ALT level (Group 1n, *n* = 19)		Initial abnormal ALT level (Group 2h, *n* = 49)	Initial normal ALT level (Group 2n, *n* = 50)	
Age, yr	54.3 ± 13.8	58.6 ± 8.3	0.222	56.6 ± 12.6	59.2 ± 9.2	0.246
Male	18 (54.6)	6 (31.6)	0.114	26 (53.1)	25 (50.0)	0.763
Initial ALT (IU/ L)	122.4 ± 125.3	26.4 ± 8.2	0.002	78.1 ± 43.9	26.8 ± 7.4	0.000
HCV RNA (log10 IU/mL)	5.6 ± 0.9	5.1 ± 1.4	0.139	6.2 ± 0.9	6.1 ± 0.7	0.748
cEVR	33 (100)	19 (100)	NA	29 (59)	29 (58)	0.906
ETR	30 (91)	18 (95)	0.626	26 (53)	30 (60)	0.491
SVR	43 (82)	40 (84)	0.83	18 (37)	22 (44)	0.466
ISHAK Score, mean ± SD						
Biopsy of receipt	17 (51.5)	14 (73.7)	0.121	33 (67.4)	30 (60)	0.453
HAI	9.6 ± 3.7	8.0 ± 3.9	0.257	8.7 ± 2.6	7.7 ± 3.0	0.164
Fibrosis score	2.9 ± 1.2	2.9 ± 1.4	0.957	3.0 ± 1.4	2.4 ± 1.4	0.100

[1]Student's *t* test. RVR: Rapid virological response; ALT: Alanine aminotransferase; HCV: Hepatitis C virus; EVR: Early virological response; ETR: End of treatment response; SVR: Sustained virological response; HAI: Histological activity index.



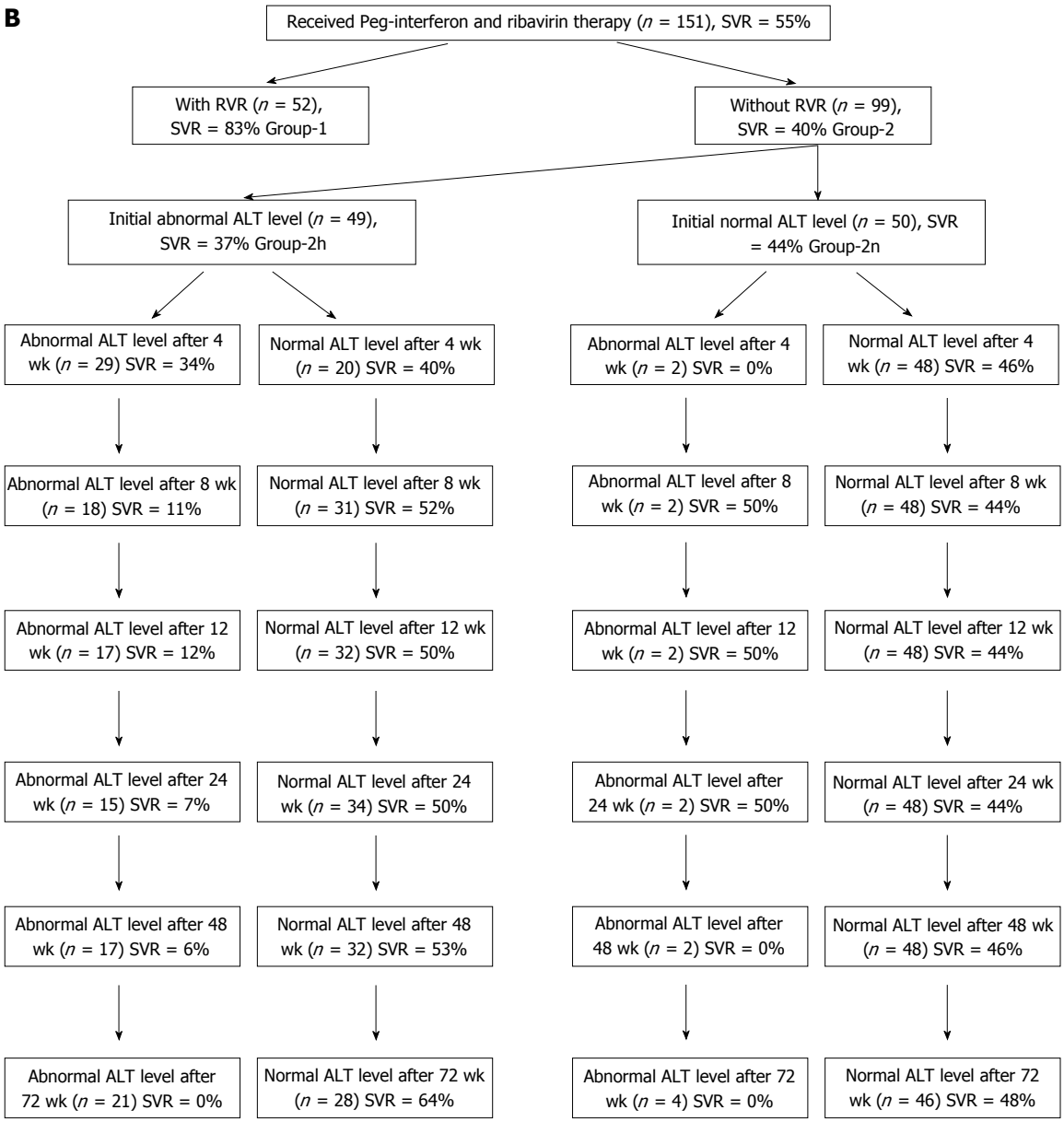


Figure 1 Schematic diagram showing patient group flow according to initial alanine aminotransferase level and subsequent pattern of change in patients with and without rapid virological response. A: Change in patients with rapid virological response (RVR); B: Change in patients without RVR. ALT: Alanine aminotransferase; SVR: Sustained virological response.

a powerful pretreatment predictor of SVR[3,4].

Once treatment is initiated, the monitoring of viral responses such RVR and early virological response (EVR) can further aid in predicting treatment response[5]. As for the response-guided approach, RVR is regarded as the most important predictor for SVR[10-12]. In a recent retrospective analysis of 1383 patients, it was shown that achieving RVR correlated with a high probability (86%-100%) of SVR to peginterferon-ribavirin combination therapy, regardless of genotype[13]. In another retrospective analysis, it was shown that the SVR rate was 42% in the absence of RVR at week 48[14]. Unfortunately, RVR rates are small and range from 7.4%-37%[15]. Also, there is a positive correlation between the magnitude of the decrease in HCV RNA at week 4 and the probability of SVR[16]. We previously demonstrated that patients with a $\geqslant$ 3 $\log_{10}$ drop in HCV RNA at week 4 have a high probability of achieving an SVR when treated with either peginterferon α-2a-ribavirin or peginterferon α-2b-ribavirin[17]. In addition, EVR is an important parameter for the decision to terminate or continue treatment because patients without EVR can hardly achieve SVR[18]. RVR seems to be the single important on-treatment factor for SVR. Consequently, there is a need for a new on-treatment predictor for SVR; especially in patients without RVR. In this context, on-treatment ALT changes may be a new predictor for SVR.

In general, a decreased pattern of ALT level is the accepted basic indicator of interferon therapeutic effect in CHC, and several studies have shown that delayed

Table 3 Comparison of sustained virological response in patients with high and normalized alanine aminotransferase levels at week 4, 8, 12, 24, 48 and 72 in patients with and without rapid virological response

	Initial abnormal ALT level in patients with RVR (Group 1h, *n* = 33)		P^2 value	Initial abnormal ALT level in patients without RVR (Group 2h, *n* = 49)		P^1 value
	Follow-up abnormal ALT	Follow-up normalized ALT		Follow-up abnormal ALT	Follow-up normalized ALT	
After 4 wk						
No. of patients	18	15		29	20	
SVR rate	78	87	0.525	34	40	0.701
After 8 wk						
No. of patients	9	24		18	31	
SVR rate	56	88	0.049	11	52	0.004
After 12 wk						
No. of patients	6	27		17	32	
SVR rate	50	89	0.028	12	50	0.007
After 24 wk						
No. of patients	5	28		15	34	
SVR rate	40	89	0.001	7	50	0.003
After 48 wk						
No. of patients	4	29		17	32	
SVR rate	25	90	0.006	6	53	0.001
After 72 wk						
No. of patients	4	29		21	28	
SVR rate	25	90	0.006	0	64	0.000

[1]Student's *t* test; [2]Student's χ^2 test. RVR: Rapid virological response; ALT: Alanine aminotransferase; SVR: Sustained virological response.

normalization of ALT levels may indicate poor response to interferon therapy[9,19], although the viral response was not always associated with biochemical response[6,20].

Serum ALT, a surrogate marker of hepatocyte damage or death, decreases during antiviral treatment, and shows the lowest activity at the end of treatment[21]. The mechanism of decline of ALT level is not clear; however it can be explained by a reduction in infected cells, a non-cytolytic cure, or cell removal irrelevant of ALT dynamics. However, a decreased ALT level at the early phase of treatment is not related to apoptotic activity[22]. Theoretically, the rapid declines in ALT may reflect a rapid decrease of ongoing inflammation in the same manner as removal of the virus. The pattern of viral elimination shows a rapid decrease in the first month. Ribeiro *et al*[21] showed that the RVR significantly correlated with the decline in ALT levels at week 4 of treatment. A retrospective study of 111 patients with chronic HCV infection treated by conventional interferon and ribavirin also demonstrated that the larger decline in ALT level within the first 2 and 4 wk was a predictor of SVR[23]. These correlations suggest that ALT dynamics can be presented as a possibility to reflect rapid virological changes; especially in patients with elevated baseline ALT levels.

Kim *et al*[24] reported that, instead of RVR, the rapid normalization of serum ALT level after initiation of treatment may play an additional role in predicting SVR. They retrospectively analyzed changes in ALT levels between baseline and week 4 of treatment in 168 patients with chronic HCV infection. Rapid normalization of ALT within 1.5 times of the normal range after treatment was found to be significantly associated with improved SVR in patients with genotype I HCV infection (34.1% *vs* 20.0%, $P = 0.01$) and non-genotype-1 infection (88.1% *vs* 66.7%, $P = 0.11$) who had initially high ALT levels. This result suggests that rapid normalization of ALT at week 4 of treatment could be used as a strategy for predicting SVR in patients with elevated baseline ALT levels; however, its use is limited because of the paucity of knowledge about RVR and the difficulty of application in normal ALT levels.

A recent report suggested that mild ALT elevations (peak ALT value 1-1.5 × baseline value) during treatment may reflect ongoing viral activity in non-responders, but a more significant rise may reflect a good virological response due to an immunomodulating effect of interferon[25]. However, it was difficult to use these data to analyze the reason for on-treatment ALT elevation and to elucidate the relationship between on-treatment ALT elevation and SVR; especially at week 4 of treatment.

In our study, patients with genotype 1 CHC were divided into two groups as those with or without RVR, because RVR is the most important on-treatment predictor of SVR. The SVR rate was also found to be high in patients with RVR (83% *vs* 40.0%, $P < 0.001$) in our study. Each group was further subdivided into two according to the initial ALT level being high or normal. The SVR rates were similar between patients with high and normal ALT levels at baseline and at week 4 in patient with and without RVR. SVR rates were found to be significantly higher in patients with normalized ALT at week 8 and thereafter.

In the patient group with RVR, SVR starts at 82% at baseline in patients with initially abnormal ALT level. SVR declines in patients with continuing abnormal ALT levels and increases in patients with normalized ALT levels. However, this difference becomes significant, with 56% *vs* 88%, only after 8 wk treatment. Later, this difference increases but at a slower rate, reaching 25% *vs* 90%

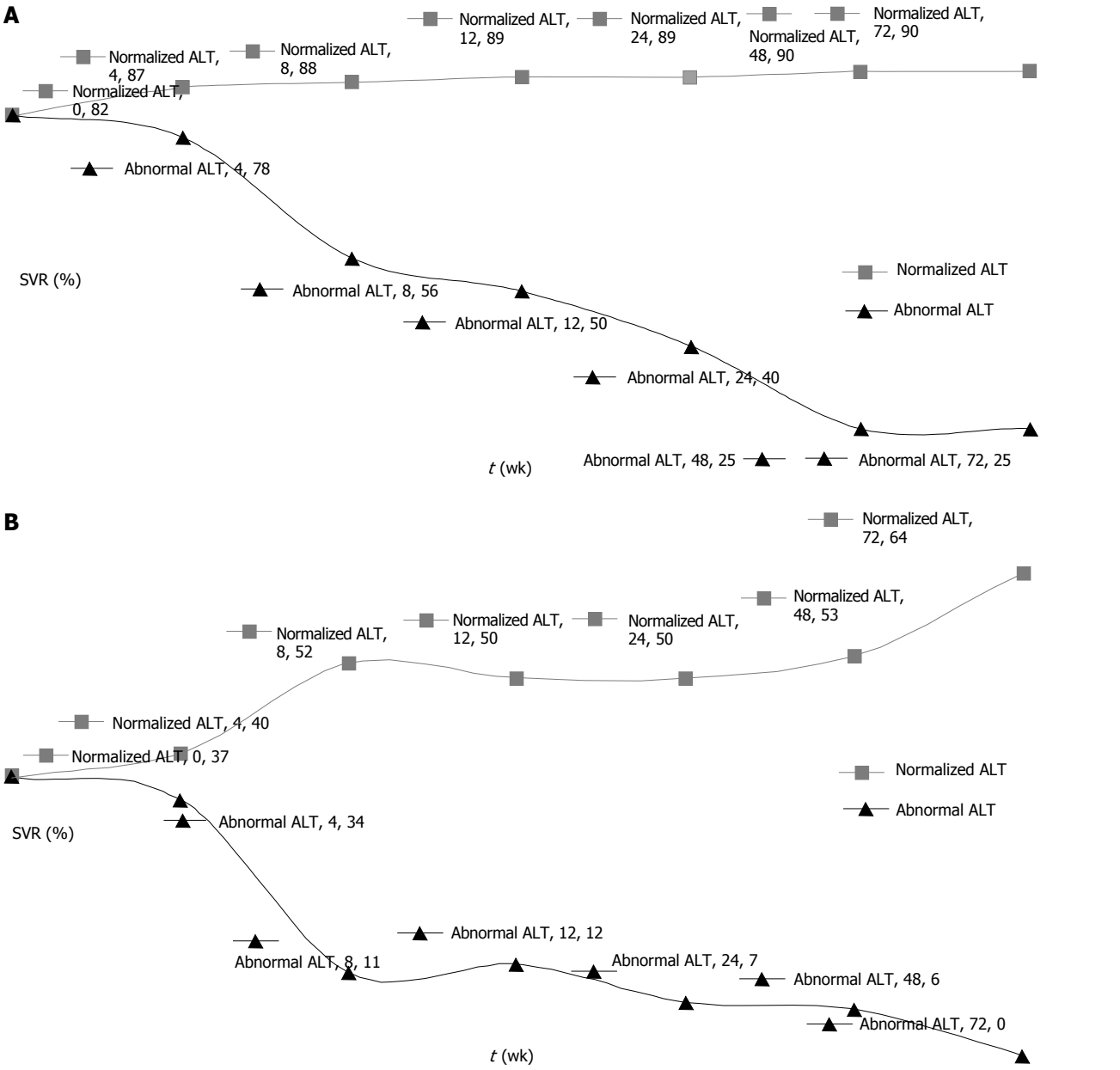


Figure 2 Sustained virological response rates in patients with follow-up abnormal and normalized alanine aminotransferase with and without rapid virological response. A: With rapid virological response (RVR); B: Without RVR. SVR: Sustained virological response; ALT: Alanine aminotransferase.

at week 48 (Figure 2A). However, it is difficult to comment on patients with continuing abnormal ALT levels because of the lower number of patients.

In the patient group without RVR, the decrease in SVR is larger in patients with continuing abnormal ALT levels. SVR starts at 37% at baseline in patients with initial abnormal ALT levels, and declines in patients with continuing abnormal ALT levels and increases in those with normalized ALT levels, as in patients with RVR. The difference in SVR levels in those groups becomes significant at 8 wk, reaching 11% *vs* 52%. The difference in SVR continues to increase slightly, reaching 6% *vs* 53% at week 48 (Figure 2B).

Although SVR was found to be significantly correlated with the decline of ALT level at week 4 of treatment in a few studies[21,23,24], high levels of ALT may also reflect a good virological response due to an immunomodulating effect of interferon[25]. Clinicians must also know the baseline ALT level in order to be able to predict SVR. Furthermore, RVR is already the most important predictor at week 4 of treatment and it is still unclear whether the use of serum ALT levels, instead of RVR, is helpful for predicting SVR in clinical practice. The main problem is to predict SVR in patients without RVR. In our study, SVR was found to be higher in patients with normalized ALT at week 4 of treatment; however, the difference was not significant at that stage (34% *vs* 40.0%, $P = 0.701$). SVR rates continued to increase and became significant at 8 wk in non-RVR patients with normalized ALT. At week 12 of treatment and later, SVR rates were found to be higher in these patients; however, cEVR was already a more important criterion for SVR at this stage, compared

to the ALT (OR = 17.55 *vs* 4.21). Therefore determination of ALT levels at 8 wk would be better than at 4 and 12 wk.

In our opinion, if a patient with initial abnormal ALT without RVR still has abnormal ALT level at 8 wk, peginterferon–ribavirin treatment may be discontinued because SVR is expected to be only 11%.

In conclusion, normalization of ALT at the 8 wk may predict viral response during peginterferon-ribavirin treatment in patients with genotype 1 CHC; especially without RVR.

COMMENTS

Background

Rapid virological response (RVR) is acknowledged as a powerful on-treatment predictor of sustained virological response (SVR) during peginterferon-ribavirin treatment of chronic hepatitis C (CHC). However, RVR rates are relatively low and a new predictor is needed for CHC patients; especially those without RVR.

Research frontiers

The authors investigated the relationship between on-treatment alanine aminotransferase (ALT) changes and SVR in patients with genotype 1 CHC during peginterferon–ribavirin treatment.

Innovations and breakthroughs

The authors found that normalization of ALT at 8 wk may predict viral response during peginterferon-ribavirin treatment in patients with genotype 1 CHC; especially without RVR.

Applications

If the patients with initial abnormal ALT without RVR still had abnormal ALT level at 8 wk, peginterferon-ribavirin treatment may be discontinued because SVR is expected to be only 11%.

Peer review

This study investigated the relationship between on-treatment ALT changes and SVR in patients with genotype 1 CHC during peginterferon-ribavirin treatment, and demonstrated that on-treatment ALT changes may be a new predictor for SVR.

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BRIEF ARTICLE

Metastatic type 1 gastric carcinoid: A real threat or just a myth?

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Abstract

AIM: To describe disease characteristics and treatment modalities in a group of rare patients with metastatic gastric carcinoid type 1 (GCA1).

METHODS: Information on clinical, biochemical, radiological, histopathological findings, the extent of the disease, as well as the use of different therapeutic modalities and the long-term outcome were recorded. Patients' data were assessed at presentation, and thereafter at 6 to 12 monthly intervals both clinically and biochemically, but also endoscopically and histopathologically. Patients were evaluated for the presence of specific symptoms; the presence of autoimmune disorders and the presence of other gastrointestinal malignancies in other family members were also recorded. The evaluation of response to treatment was defined using established WHO criteria.

RESULTS: We studied twenty consecutive patients with a mean age of 55.1 years. The mean follow-up period was 83 mo. Twelve patients had regional lymph node metastases and 8 patients had liver metastases. The primary tumor mean diameter was 20.13 ± 10.83 mm (mean ± SD). The mean Ki-67 index was 6.8% ± 11.2%. All but one patient underwent endoscopic or surgical excision of the tumor. The disease was stable in all but 3 patients who had progressive liver disease. All patients remained alive during the follow-up period.

CONCLUSION: Metastatic GCA1 carries a good overall prognosis, being related to a tumor size of ⩾ 1 cm, an elevated Ki-67 index and high serum gastrin levels.

Key words: Metastatic gastric carcinoids; Gastrin; Chromogranin A; Somatostatin analogues; Stomach neuroendocrine tumor

Core tip: Metastatic gastric carcinoid type 1 (GCA1) are extremely rare and there is no data regarding their natural history, treatment and prognosis. Based on our study, metastatic GCA1 carries a good overall prognosis. Metastatic spread appears to be related to a tumor size of ⩾ 1 cm, an elevated Ki-67 index, and to high serum gastrin levels. Endoscopic surveillance is extremely important for follow-up. Surgical resection should be performed only in patients in whom total tumor excision is expected. Although still controversial, somatostatin analogues could be considered as first line treatment to lower the elevated gastrin levels and suppress enterochromaffin like cell hyperplasia.

Grozinsky-Glasberg S, Thomas D, Strosberg JR, Pape UF, Felder S, Tsolakis AV, Alexandraki KI, Fraenkel M, Saiegh L, Reissman P, Kaltsas G, Gross DJ. Metastatic type 1 gastric carcinoid: A real threat or just a myth? *World J Gastroenterol* 2013; 19(46): 8687-8695 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i46/8687.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i46.8687

INTRODUCTION

Gastric carcinoids (GCAs) are neuroendocrine tumors (NETs) of the gastric mucosa originating from enterochromaffin like (ECL) cells[1]. GCAs arise either spontaneously or in response to a chronic hypergastrinemia state; due to their rarity (only 2% of all carcinoids and 8.7% of gastrointestinal carcinoids)[2,3], the predictors of metastatic disease have not been systematically addressed.

GCAs are divided into three distinct types. Type 1 (GCA1) represents the majority (approximately 75%) and is associated with chronic atrophic gastritis and autoimmune destruction of parietal cells. Type 2 (GCA2) (approximately 5%-10%) occurs almost always in the context of Multiple Endocrine Neoplasia type 1 (MEN1). Both types 1 and 2 GCAs occur in the setting of elevated serum gastrin which exerts a trophic effect on gastric enterochromaffin-like (ECL) cells leading to neuroendocrine cell hyperplasia and multifocal polypoid carcinoid tumors. These tumors are well differentiated and carry an excellent overall prognosis. Type 3 GCAs (15%-25%) are not related to hypergastrinemia and follow an aggressive course[4-6].

Type 1 GCAs are usually discovered during upper gastrointestinal tract (GIT) endoscopy performed for non specific symptoms (nausea, abdominal pain, dyspepsia)[7], or during investigation of anemia[8-10]. In the past, type 1 GCA was frequently diagnosed in women in their 5th to 7th decades; however, with the more extensive use of endoscopy, the diagnosis occurs at a younger age[11].

Traditionally, GCA1s are endoscopically removed[12,13]; antrectomy could be considered to remove the source of excessive gastrin secretion[14]. Importantly, somatostatin analogues (SSAs) have been increasingly used in the treatment of patients with GCA1 or GCA2[15], based on their capability to inhibit gastrin release, reduce the ECL cell hyperplasia[16-20], and to substantially decrease tumor load[21-23].

Metastatic GCA1 are extremely rare and little is known about their natural history, treatment and prognosis. We conducted a multicenter, retrospective analysis to describe disease characteristics and treatment modalities in a group of rare patients with metastatic GCA1.

MATERIALS AND METHODS

Twenty consecutive patients with metastatic GCAs1 treated in five tertiary referral centers for at least 6 mo were studied. Information on clinical presentation, biochemical profile, imaging, histopathological findings and disease extent (using the TNM classification)[24] were recorded. The use of varying therapeutic modalities and the long-term outcome of these patients were also recorded. Patients' data were assessed at presentation, and thereafter at 6-12 monthly intervals both clinically and biochemically, but also endoscopically and histopathologically.

Clinical assessment

Patients were evaluated for the presence of symptoms such as abdominal pain, nausea, vomiting and dyspepsia; the presence of autoimmune disorders associated with pernicious anemia and the presence of other gastrointestinal malignancies in other family members were also recorded.

Biochemical evaluation

Pernicious anemia was defined as a low serum vitamin B_{12} level (normal range 180-670 pmol/L) and at least one positive antibody against parietal cells, intrinsic factor or proton-pump antigen. Serum gastrin and chromogranin A (CgA) were measured after an overnight fast, and thereafter at regular intervals (3-6 mo) during the study period. Treatment with proton pump inhibitors (PPIs) was discontinued for at least 3 wk before blood samples were taken. Serum CgA and gastrin were measured using commercially available radioimmunoassay kits: CGA-RIACT, CISBIO International, France (normal reference range of 19.4-98.1 ng/mL), or Euro-Diagnostica, Malmö (upper normal limit 4 nmol/L) for CgA, and DiaSorin, Stillwater, Minnesota 55082-0285, United States (normal reference range of 40-108 mU/L) or EURO-Diagnostica, Malmö (upper normal limit 60 pmol/L) for gastrin, respectively.

Imaging assessment

All patients underwent imaging assessment at diagnosis, including either ^{111}In-pentetreotide scintigraphy (Octreoscan) or (68)Gallium-DOTA-TATE/-TOC/-NOC

Table 1 Clinical and histopathological characteristics of the study patients

Patient No.	No. of lesions	Size, largest lesion (mm)	Ki-67%	CT before Tx	SRS/^{68}Ga before Tx	Distant mets	Gastrin (40-108 mU/L)		Surgery	Residual disease	SSA/monthly dosage (mg)	Outcome	F/U Period (mo)
							at Dx	Last					
1	multiple	15	2%	-	Uptake (stomach, LN)	no	1811	125	wedge resection	no	Som A 90	cure	108
2	solitary	35	2%	Liver mets	Uptake (stomach, Liver)	liver	-	-	Billroth 2 + LN	liver	no	SD	84
3	multiple	21	2%	Liver mets	Uptake (stomach, Liver)	liver	2204	325	none	liver	San LAR 30	CR	36
4	multiple	55	20%	Stomach lesion	Uptake (stomach)	diaphragm	1800	-	Billroth 2 + LN	no	San LAR 30	cure	24
5	multiple	25	1%	Stomach lesion	Uptake (stomach, LN)	no	1403	-	Billroth 2 + LN	no	no	cure	18
6	multiple	-	15%	Hepato-gastric ligament LN	no data	no	-	-	ER	no	no	cure	12
7	solitary	17	4%	LN	Uptake (stomach, LN)	no	700	600	wedge resection	no	no	cure	132
8	multiple	15	1%	Liver mets and LN	Uptake (LN, Liver)	liver	407	190	ER, largest	liver	San LAR 30 + INF 50 mcg/wk	PR	36
9	solitary	10	1%	Liver mets and LN	no data	liver	-	-	wedge resection	no	no	cure	360
10	solitary	30	5%	Stomach lesion	no uptake	no	5130	43	Billroth 2 + LN	no	none	cure	120
11	multiple	17	1%	-	no uptake	no	-	-	Billroth 2 + LN	no	none	cure	168
12	multiple	14	5%	-	no uptake	no	-	-	Billroth 2 + LN	no	none	cure	72
13	solitary	47	15%	-	Uptake (stomach, Liver)	liver	5470	45	ER	liver	none	SD	120
14	multiple	30	2%	-	Uptake (LN, Liver)	liver	1336	335	ER, recurrent	liver	none	SD	48
15	multiple	10	2%	No pathology	no uptake	no	3500	-	Billroth 2 + LN	no	none	cure	48
16	multiple	30	2%	Stomach lesion	no data	no	-	-	Billroth 2 + LN	no	none	cure	36
17	solitary	-	1%	Liver mets	Uptake (liver)	liver	1612	10	Billroth 2 + LN	liver	none	SD	36
18	multiple	20	-	Stomach lesion, mesenteric and gastro-hepatic LN	no data	no	506	-	wedge resection	no	none	cure	60
19	multiple	20	-	No pathology	no uptake	no	1600	-	Billroth 2 + LN	no	none	cure	72
20	multiple	15	-	Liver mets	Uptake (stomach, Liver)	liver	458	336	ER	liver	San LAR 30	SD	72

Nr. of lesions, solitary: one lesion seen on endoscopy, multiples ⩾ 2 lesions seen on endoscopy; CT: Computerized tomography; SRS: Somatostatin receptor scintigraphy (Octreoscan); (68)Ga: (68)Gallium-DOTA-TATE/-TOC/-NOC PET; Tx: Treatment; SSAs: Somatostatin analogues; LN: Lymphadenopathy; mets: Metastases; SomA: Somatuline Autogel; SanLAR: Sandostatin LAR; INF: Interferon α; Billroth 2 + LN: Gastro-jejunostomy and lymph nodes dissection; wedge resection: wedge resection (triangular resection) of a part of the stomach; ER: Endoscopic resection; SD: Stable disease; PR: Partial response; CR: Complete response.

PET (17 patients), computerized tomography (CT) of the abdomen (13 patients), or both modalities (11 patients) (Table 1).

Endoscopic and histopathological assessment

All patients underwent upper GI endoscopy and 6/20 also underwent endoscopic ultra-sonography (EUS). Upper GI endoscopy with multiple biopsies was performed in order to assess the lesions and map surrounding gastric mucosa for changes of atrophic gastritis; the "dominant" lesions were biopsied and removed if possible. EUS was performed to assess invasion of the muscularis propria, regional lymph node involvement and/or visible metastases. Histopathological diagnosis was performed using biopsies taken from both the tumors and the surrounding mucosa at diagnosis or periodically during the follow-up period, or, in case of tumor excision - from the surgical specimen. Sections were immunostained for chromogranin (CG), neuron specific enolase (NSE), synaptophysin (SYN), and the Ki-67 proliferative index using the MIB-I antibody. The diagnosis of NETs was confirmed morphologically during endoscopy together with a positive immunocytochemical staining for NSE, SYN and/or CG.

Table 2 Factors of significance in the suspicion of metastatic gastric carcinoid type 1 *n* (%)

Characteristics	All GCA1 patients (*n* = 254)	Metastatic GCA1 patients (*n* = 20)	*P* value at diagnosis (metastatic *vs* all GCA1)
Age (yr), mean ± SD	58.5 ± 12.7	55.1 ± 12.8	0.050
Size of largest tumor (mm, mean ± SD)	7.9 ± 12.1	20.14 ± 11	< 0.001
Ki-67 (%, mean ± SD)	1.9 ± 2.4	6.8 ± 11.2	< 0.001
Symptomatic	112 (44)	18 (90)	< 0.001
Gastrin levels (mI/L, mean ± SD) at diagnosis	898 ± 418	2138.4 ± 1562	< 0.001

GCA1: Gastric carcinoid type 1.

Table 3 Demographic and clinical characteristics of the patients included in the study *n* (%)

Characteristics	All patients *n* = 20
Age (yr), mean ± SD	55.1 ± 12.8
Male:female, *n*	9:11
Caucasians	95%
Size of primary tumor (mm), mean ± SD	20.14 ± 11
Symptomatic	18 (90)
Atrophic gastritis	20 (100)
Other autoimmune diseases	2 (10)
Familial aggregation	3 (15)

Evaluation of response to treatment

Disease response was defined using established WHO criteria[24].

Patients were considered in remission if symptoms disappeared, gastrin and CgA levels were substantially reduced (> 50% reduction) or returned to normal range and if there was no evidence of residual disease following treatment. The study was approved by the local institutional ethical committees and informed consent was obtained from all patients.

Statistical analysis

Results were expressed as mean ± SD. Nonparametric ANOVA (Kruskal-Wallis one-way ANOVA) was used to assess and compare different parameters (such as the mean age at diagnosis, the size of the largest tumor, the Ki-67 *etc*.) at diagnosis (Table 2), or the levels of gastrin at diagnosis and following surgical treatment/at last visit (Table 1). Post hoc comparisons were made using Mann-Whitney *U* test. A *P* value of < 0.05 was considered significant.

RESULTS

The clinical characteristics of all patients included in the study are shown in Table 3. The cohort included 9 men and 11 women with a mean age of 55.1 years. Whereas women are usually at higher risk for autoimmune atrophic gastritis, our cohort included patients of both genders, showing only a slight preponderance in the number of female patients. The mean duration of follow-up was 83 mo (range 12-360 mo). Other autoimmune diseases (*e.g.*, Hashimoto's thyroiditis, Sjögren's syndrome) were diagnosed in two patients (10%). In three patients (15%) there was a first-degree relative with history of gastric (2 patients) or pancreatic (one patient) adenocarcinoma.

Basal evaluation (at diagnosis)

At diagnosis gastroscopy revealed macroscopic gastric carcinoid tumors (described as "nodules", "ulcers" or "polyps") in all patients, with a mean diameter of 20.13 ± 10.83 mm (mean ± SD) (range 4-55 mm). The tumors were single in 6/20 patients (30%), and multiple (defined as ⩾ 2 tumors seen on gastroscopy) in the remaining 14 (70%). ECL cell hyperplasia was observed in all patients. The mean Ki-67% proliferation index was 6.8% ± 11.2% (range 1%-20%). None of the patients included in the present series presented with ZES and the associated MEN1 syndrome or with characteristics of type 3 gastric carcinoids (Tables 1 and 4).

EUS was intended to be performed in all patients in order to reveal any residual and/or sub-mucosal tumors. Signs of aggressiveness or invasiveness at first biopsy were demonstrated in seven out of 12 patients with available data (58%) and included: ulceration of the primary lesion in two patients (17%); vascular invasion in two patients (17%); invasion of the muscularis mucosa and lamina propria in four patients (33%). Peri-gastric/gastro-hepatic ligament lymph node invasion was observed in 9 patients (45%) as demonstrated by CT scan and/or Octreoscan or (68)Ga-DOTATOC/NOC/TATE PET-CT; distant metastases were present at initial diagnosis in 9 patients (45%), and included liver metastases in eight and diaphragmatic metastases in one out of the 20 patients.

Treatment

Ten out of the twenty patients (50%) underwent total gastrectomy or a Billroth 2 operation (gastro-jejunostomy) and lymph node dissection, another 4 patients (20%) underwent antrectomy and wedge resection, whereas endoscopic resection of the dominant lesion was performed in 5 patients (25%). One patient underwent only primary tumor biopsy (Table 1, patient No. 3).

Histopathological analysis following tumor resection demonstrated positive staining by immunohistochemistry (IHC) for neuroendocrine markers (chromogranin and synaptophysin) in all patients (100%), for vesicular monoamine transporter 2 (VMAT2) in two patients (10%), and for neuron specific enolase (NSE) in seven patients (35%). Ki-67 indices were available in 17 out of the 20 patients included; eleven tumors were defined as ENETS grade 1

Table 4 Features associated with the diagnosis of gastric carcinoid type 1 in our patients

Patient No.	Vitamin B12 levels (n. 180-670 pmol/L)	APCA	Gastrin levels (n. 40-108 mU/L)	Prior use of PPIs	1st gastroscopy (macroscopic)	Histo-pathology	*H.Pylori*
1	45	positive (1/20)	1811	no	multiple	CAG + IM	negative
2	165	positive	-	no	solitary	CAG + IM + NECH	-
3	333	positive (1:160)	2204	no	multiple	CAG + NECH	negative
4	186	positive (1:20)	1800	no	multiple	CAG + NECH	negative
5	104	positive (1:20)	1403	no	multiple	CAG + NECH	negative
6	122	positive (1:80)	-	no	multiple	CAG	-
7	121	-	700	no	solitary	CAG + IM + NECH	-
8	86	-	407	no	multiple	CAG + IM + NECH	-
9	50	-	-	no	solitary	CAG + NECH	-
10	-	positive (1:40)	5130	no	solitary	CAG	-
11	-	-	-	no	multiple	CAG	-
12	-	-	-	no	multiple	CAG	-
13	184	positive (1:160)	5470	no	solitary	CAG	-
14	121	positive	1336	no	multiple	CAG	-
15	215	-	3500	no	multiple	CAG	-
16	-	-	-	no	multiple	CAG + IM + NECH	-
17	345	-	1612	no	solitary	CAG	-
18	130	-	506	no	multiple	CAG	-
19	181	-	1600	no	multiple	CAG	negative
20	167	positive	458	no	multiple	CAG	-

APCA: Antiparietal cells antibodies; PPIs: Proton pump inhibitors; CAG: Chronic atrophic gastritis; IM: Intestinal metaplasia; NECH: Neuroendocrine cells hyperplasia; *H. pylori*: *Helicobacter pylori*.

(Ki-67 ⩽ 2%) and six tumors as grade 2 (Ki-67 between 2%-20%). The final value for the mean Ki-67 proliferation index measured 4.8%, slightly lower than the Ki-67 value at first endoscopy (6.8%); interestingly, the Ki-67 was significantly higher in the liver/lymph node metastases than in the primary tumor in 4/20 patients.

Based on local team decision, five out of the 20 patients assessed were treated with somatostatin analogues (SSAs): in four patients Sandostatin LAR (Novartis, Basel, Switzerland) 30 mg/month, in one patient Somatuline Autogel (Ipsen, Paris) 90 mg/month, whereas in one patient pegylated interferon alpha was added to the SSA at a dosage of 50 micrograms per week, as anti-secretory and anti-proliferative therapy.

Treatment related adverse events were reported in only 3 patients and included diarrhea (one patient), fatigue (in the patient treated with interferon alpha) and gastrectomy-related dumping syndrome in one patient.

None of the patients received chemotherapy or peptide receptor radioligand therapy, to date.

Laboratory and imaging assessment at diagnosis

Gastrin and CgA levels were elevated at diagnosis in all patients with available data (14/20 patients for gastrin, and 13/20 patients for CgA) and reached 2138.4 ± 1562 mU/L for gastrin (normal range 40-108 mU/L) and 507.6 ± 403.7 ng/mL for CgA (normal range 19.4-98.1 ng/mL), respectively. No clear correlation was found between initial gastrin and CgA serum levels and the number or size of the tumors.

High levels of anti-parietal cells antibodies were found in all patients in whom their titer was determined. The levels of vitamin B_{12} were low in all but six patients, with a mean value of 162 ± 87 pmol/L (normal range 180-670 pmol/L) (Table 4).

Data on functional imaging - ^{111}In-pentetreotide scintigraphy (Octreoscan) or (68)Ga-DOTATOC/NOC/TATE PET-CT (performed based on local availability) were available at diagnosis in 17/20 included patients: in 12 patients (71%) there was increased tracer uptake by the gastric lesions as well as by the perigastric metastatic lymph nodes and liver lesions. Twelve patients underwent (68)Ga-DOTATOC/NOC/TATE-PET-CT demonstrating an increased uptake by the tumor and metastases in 9 patients, and no pathological uptake in the remaining 3 patients. Five patients performed an Octreoscan, showing increased uptake by the tumor in 3, and no pathological uptake in 2.

Interestingly, in the five patients with no pathological uptake by either functional imaging method, the Ki-67 index of proliferation was ⩽ 2% and the tumor size was > 1 cm.

Follow-up assessment and treatment outcome

All patients remained alive during the follow-up period. During follow-up after the first intervention, the disease was stable in all patients: in the subgroup who underwent total gastrectomy or Billroth 2 operation (gastro-jejunostomy) and lymph node dissection (10 patients, 50%), as well as in the subgroup of the 4 patients (20%) who underwent antrectomy and wedge resection, the disease did not progress or recur during follow-up. The same was observed in the other patients in the present series, including those who underwent repeated endoscopic resection of the largest lesions. In the seven patients with persistent liver disease, somatostatin analogue treatment was administered in three patients: in two Sandostatin LAR 30 mg/month alone, (inducing disease stabilization in

one patient and complete response in the other), whereas in the third patient pegylated interferon α (PegIntron) at a dosage of 50 micrograms/week was added to Sandostatin LAR 30 mg/month, and induced partial response of the liver metastases. All patients tolerated treatment with SSAs well and none discontinued treatment during the follow-up period. Apart from a slight perturbation in the control of pre-treatment diabetes mellitus in one patient (Table 1, patient 3), there were no other adverse effects associated with somatostatin analogue treatment. Eighteen patients (90%) had symptoms attributed to the disease (such as abdominal pain, nausea, vomiting or dyspepsia) that improved in all following treatment.

Serum gastrin decreased progressively in all patients with available data, from 2138.4 ± 1562 mI/L pre-treatment to 223 ± 193 mI/L at the last visit (normal range 40-108 mI/ L, $P < 0.005$). The levels of serum CgA also significantly decreased, from 507.6 ± 403.7 ng/mL to 57 ± 44.7 ng/mL (mean ± SD) (normal range 19.4-98.1 ng/mL, $P < 0.005$).

DISCUSSION

GCAs are rare neoplasms, accounting for about 1.25% of all malignancies[25]. Their incidence, however, is increasing, most probably as result of the widespread use of endoscopy and imaging. Despite the relatively indolent biological behaviour of GCA1 tumors, approximately 8%-23% have been reported as presenting with an aggressive clinical course, metastasizing to regional lymph nodes and rarely to the liver[7].

The European Neuroendocrine Tumor Society (ENETS) consensus guidelines on GCA1 treatment are based on tumor size (less or more than 1 cm) and specify that, despite a preference for a conservative approach, based on endoscopic follow-up, lesion resection is recommended whenever possible[26]. Specifically, in patients with lesions of more than 1 cm, EUS should be performed to assess gastric wall and lymph nodal involvement before the decision about the type of excision (endoscopic mucosal resection, EMR, or subtotal gastrectomy/wide resection) is taken. Although biotherapy with somatostatin analogues (SSAs) is still a matter of debate according to the ENETS guidelines, we and others have recently demonstrated the beneficial effect of long acting SSAs monthly administration on inhibition of gastrin and CgA levels and of tumor progression, as shown from the regression of ECL-cell hyperplasia and tumor disappearance observed in the great majority of treated patients[21,27,28]. The combination of octreotide and α-interferon has been also reported to be of value in a patient with metastatic disease to the liver[7].

As the therapeutic modalities to inhibit tumor progression in metastatic GEP-NETs are still unsatisfactory, new approaches are under investigation. Recent preclinical data demonstrated possible beneficial effects of interferon-beta (IFN-β) in inhibiting cell proliferation and stimulating apoptosis in a PNET cell line model[29-31]. Moreover, a new gastrin/CCK2 receptor antagonist molecule, YF476, appears to induce potent inhibition of ECL cell proliferation compared with dopamine agonists or dopamine/somatostatin chimera molecules, and to provide new insights for the therapy of hypergastrinemic gastric NETs associated with low acid states, such as in our patients[32]. Noteworthy, a recent phase Ⅱ study demonstrated good tolerability for the multi receptor ligand SSA pasireotide (SOM230) in patients with GEP NETs refractory to available SSAs[33].

In the present study we sought to define risk factors for increased malignant potential at the time of diagnosis in patients with GCA1. From a total of 254 consecutive patients with GCA1 followed and treated at 5 tertiary referral medical centers, we identified 20 patients with metastatic disease to locoregional lymph nodes or liver at presentation (7.9%). In our series, the patients with metastatic GCA1 were younger, had larger tumors, had a higher Ki-67 proliferation index, and presented with higher gastrin levels compared with the group of patients with non-metastatic GCA1 tumors (Table 2). These results are in accordance with a recent study published by Saund MS and coworkers[34], demonstrating that in a group of 984 patients with localized GCA1, tumor size and depth predict lymph node metastasis; they recommended endoscopic resection for intraepithelial tumors < 2 cm and perhaps tumors < 1 cm invading into the lamina propria or submucosa.

In the present series, most of the patients with metastatic GCA1 were symptomatic, with presence of epigastric or abdominal pain, dyspepsia, bloating, nausea, loose stools or early satiety. A possible explanation for these symptoms may be the presence of atrophic gastritis together with achlorhydria in all patients with GCA1, as well as the increased levels of gastrin[35,36].

Of note, there was a clear correlation between the size of the tumor at diagnosis and tumor metastatic spread in our study, as in all patients included the tumor size was ⩾ 1 cm. Moreover, the mean Ki-67 index of proliferation in the metastatic GCA1 was significantly higher than in the localized tumors (Table 2), most probably due to an increased number of patients with grade 2 tumors in our series (6/20 patients, 30%) and indicating the utmost importance of performing immunohistochemical staining for this marker in all patients with GCA1. Findings of aggressiveness and/or invasiveness at diagnosis (*e.g.*, ulceration of the lesion, vascular invasion, muscularis propria or lamina propria invasion) are all predictive factors for an aggressive biological behaviour, in parallel with a tumor size of ⩾ 1 cm. In this high risk group, EUS or cross-sectional imaging should be performed to assess the presence of lymph nodes/liver metastatic disease.

Regarding the imaging characteristics of metastatic GCA1, it appears from our study that no radiological parameters, tumor number or tumor uptake on somatostatin receptor scintigraphy could distinguish between local and metastatic tumors. All of the metastatic GCA1 patients accomplished tumor resection with a low compli-

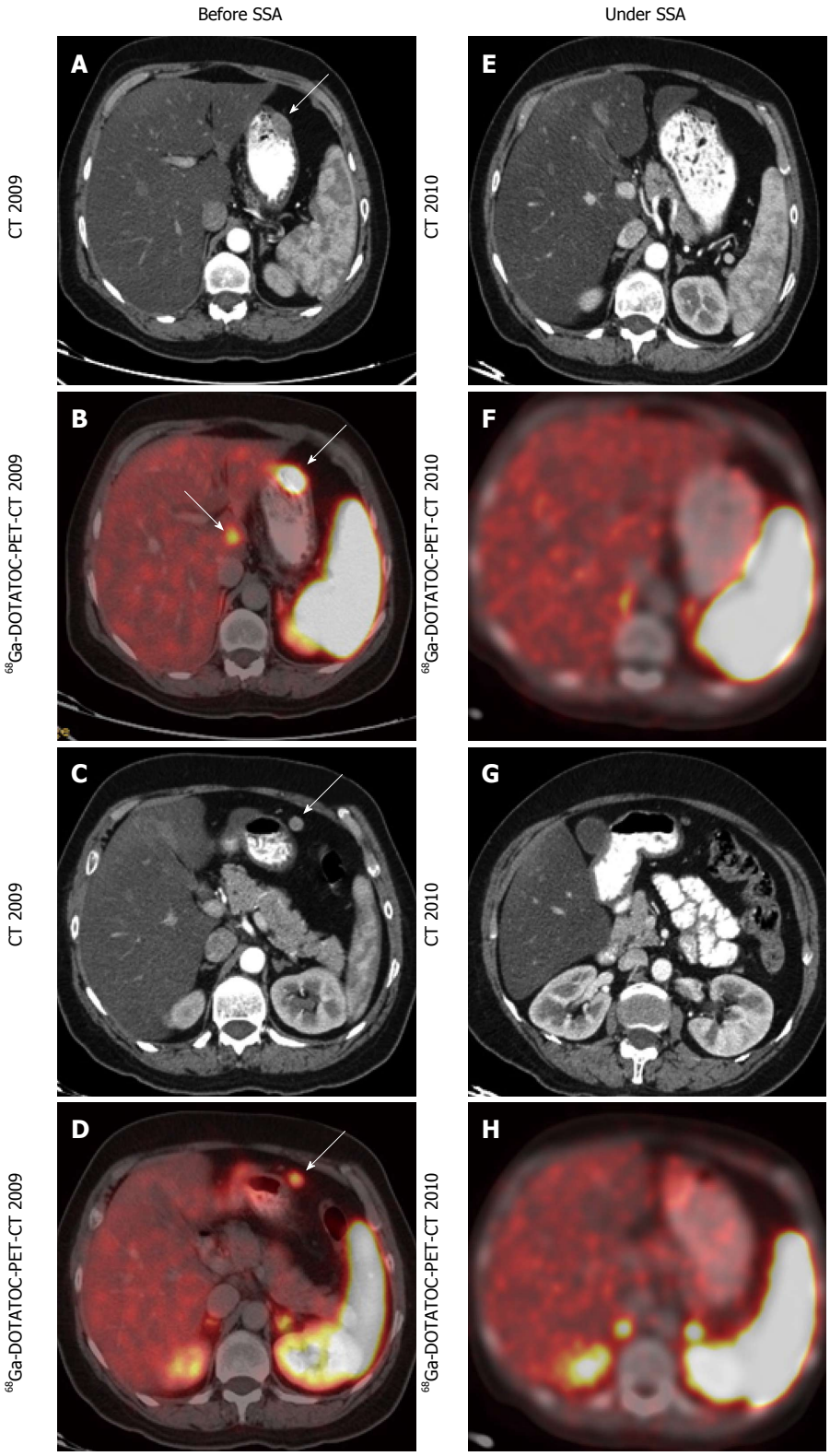

Figure 1 Computed tomography and ^{68}Ga-DOTATOC-PET-Computed tomography images before and during treatment with somatostatin analogue (sandostatin LAR 30 mg/mo). Pathologic uptake in the gastric and hepatic lesion (A + B) adjacent lymphadenopathy and liver lesion (C + D), disappeared on follow up imaging (E - H).

cation rate, and with an excellent outcome. Following or in parallel with tumor resection, medical therapy was administered in five patients, based on clinical experience. Importantly, under treatment with SSAs, the disease stabilized in 3 patients, in one patient the primary tumor, the metastatic lymph nodes and the liver metastases regressed and completely disappeared (Figure 1 and Table 1), whereas in another patient, pegylated interferon α

was added to the SSA and induced disease stabilization. In none of the twenty patients with metastatic GCA1 was disease progression observed over a mean follow-up period of 54 mo.

Based on the results of our study, metastatic GCA1 do exist, are extremely rare, and carry a good overall prognosis. Metastatic spread appears to be related to a tumor size of ⩾ 1 cm, and therefore endoscopic ultrasound evaluation is recommended in such patients. Elevated Ki-67 index of tumor proliferation, as well as high serum gastrin levels, represent additional risk factors for metastatic disease. Endoscopic resection and/or subtotal gastrectomy are recommended by the ENETS guidelines in all patients with gastric carcinoids of ⩾ 1 cm; however, in our personal opinion[21], SSAs might be considered as possible treatment in order to lower the elevated gastrin levels, suppress ECL cell hyperplasia, and obviate the need for surgical excisions, particularly in patients with multiple or relapsing tumors, as well as in those with metastatic disease of the liver. Treatment with SSAs could be theoretically continued as long as gastrin/CGA levels are suppressed, in parallel with disease stabilization observed on regular endoscopic follow-up. However, this approach is still problematic by the lack of controlled trials, the high cost of these drugs as well as the limited accessibility to SSAs in some areas. Although the potential role of SSAs ("cold" SSAs, as monthly injections, or radioactive "hot" SSAs, PRRT) cannot be denied - it remains still controversial and it has to be confirmed in larger studies. Moreover, surgical procedures should be most probably performed only in patients in whom total tumor excision can be expected. Therefore, in these patients, endoscopic surveillance (as well as repeated oncological surveillance by imaging in metastatic cases) is the most important measure. Prospective multicenter randomised studies, including larger number of patients, would be optimal for definition of the best therapeutic approach, the duration of treatment and its efficacy in terms of long-term survival. However, due to the extreme rarity of this condition, the probability for such trials is remote, and therefore clinicians who manage these patients will most probably have to rely on personal experience and data from retrospective studies, such as ours.

COMMENTS

Background

Gastric carcinoids (GCAs) are rare neuroendocrine tumors (NETs) of the gastric mucosa originating from enterochromaffin like (ECL) cells. Type 1 (GCA1) represents the majority, and usually carries an excellent overall prognosis.

Research frontiers

Metastatic GCA1 are extremely rare and little is known about their natural history, treatment and prognosis. The present study represents a multicenter, retrospective analysis aiming to describe disease characteristics and treatment modalities in a group of rare patients with metastatic GCA1.

Innovations and breakthroughs

The authors demonstrated that the metastatic potential of GCA1 appears to be related to a tumor size of ⩾ 1 cm, an elevated Ki-67 index and high serum gastrin levels. Endoscopic ultrasound is recommended in patients with these risk factors. Somatostatin analogues may be used, particularly in patients with multiple relapsing tumors, and with metastatic disease. Surgical procedures should be performed only in patients in whom total tumor excision is expected.

Applications

By understanding the potential malignant behavior of these rare tumors, this study may represent a future strategy for therapeutic intervention in patients with metastatic GCA1.

Peer review

This is a useful multicenter, retrospective analysis of a rare disease and provides helpful information on risk factors, tumor characteristics, treatment procedures and prognosis in a wide and rare group of patients with metastatic GCA1.

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BRIEF ARTICLE

Risk factors to predict severe postoperative pancreatic fistula following gastrectomy for gastric cancer

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Abstract

AIM: To allow the identification of high-risk postoperative pancreatic fistula (POPF) patients with special reference to the International Study Group on Pancreatic Fistula (ISGPF) classification.

METHODS: Between 1997 and 2010, 1341 consecutive patients underwent gastrectomy for gastric cancer at the Department of Digestive Surgery, Kyoto Prefectural University of Medicine, Japan. Based on the preoperative diagnosis, total or distal gastrectomy and sufficient lymphadenectomy was performed, mainly according to the Japanese guidelines for the treatment of gastric cancer. Of these, 35 patients (2.6%) were diagnosed with Grade B or C POPF according to the ISGPF classification and were treated intensively. The hospital records of these patients were reviewed retrospectively.

RESULTS: Of 35 patients with severe POPF, 17 (49%) and 18 (51%) patients were classified as Grade B and C POPF, respectively. From several clinical factors, the severity of POPF according to the ISGPF classification was significantly correlated with the duration of intensive POPF treatments (P = 0.035). Regarding the clinical factors to distinguish extremely severe POPF, older patients (P = 0.035, 65 years $\leqslant$ *vs* < 65 years old) and those with lower lymphocyte counts at the diagnosis of POPF (P = 0.007, < 1400/mm^3 *vs* 1400/mm^3 $\leqslant$) were significantly correlated with Grade C POPF, and a low lymphocyte count was an independent risk factor by multivariate analysis [P = 0.045, OR = 10.45 (95%CI: 1.050-104.1)].

CONCLUSION: Caution and intensive care are required for older POPF patients and those with lower lymphocyte counts at the diagnosis of POPF.

Key words: Pancreatic fistula; International Study Group on Pancreatic Fistula classification; Gastric cancer; Gastrectomy; Complication

Core tip: Although several possible risk factors associated with the occurrence of postoperative pancreatic fistula (POPF) have been reported, there have been no generally accepted risk factors to predict POPF changing into extremely severe POPF. In this study, we demonstrated that older patients (P = 0.035) and those with lower lymphocyte counts at the diagnosis of POPF (P = 0.007) were significantly associated with extremely severe International Study Group on Pancreatic Fistula grade C POPF, and a low lymphocyte count was identified as an independent risk factor by multivariate analysis (P = 0.045, OR = 10.45).

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INTRODUCTION

Recent advances in less invasive treatment techniques and the perioperative management of gastric cancer have decreased the mortality and morbidity rates associated with this disease[1,2]. However, postoperative pancreatic fistula (POPF) is still a major complication following gastrectomy. Once POPF develops, it sometimes contributes to lethal complications, such as abdominal abscesses, secondary anastomotic leakage, and intra-abdominal hemorrhage.

Many surgeons have previously reported several possible risk factors for the occurrence of POPF. It has been reported that the incidence of POPF associated with surgical procedures is higher following radical or extended lymphadenectomy[3,4], splenectomy, or pancreaticosplenectomy[5-7]. Host-related factors on POPF have also been clarified, in which the occurrence of POPF has been significantly correlated with a higher body mass index (BMI) and visceral fat area (VFA), being male, hyperlipidemia, and comorbidities[8-11].

Thus, to decrease the incidence of POPF, several clinical studies have been performed with the aims of avoiding unnecessary surgery and standardizing surgical procedures[4,12]. Moreover, in order to lower the risk of tissue damage and make surgical procedures easier, surgical devices such as ultrasonic activated coagulating scalpels have been developed and most surgeons are currently cautious of the risk factors associated with POPF. However, to date, after patients develop POPF, there are no generally accepted risk factors to predict the change to severe POPF in these patients. Indeed, indicators that provide an objective description of the patient's condition at specific points in the disease process of POPF are useful to improve understanding of the complications that may be encountered.

In this study, we confirmed that the severity of POPF according to the International Study Group on Pancreatic Fistula (ISGPF) classification was correlated with the duration of intensive POPF treatments. Furthermore, we clarified an independent risk factor to predict the worst outcome of POPF treatment with special reference to the ISGPF classification.

MATERIALS AND METHODS

Patients and surgical procedures

Between 1997 and 2010, 1341 consecutive patients underwent gastrectomy for gastric cancer at the Department of Digestive Surgery, Kyoto Prefectural University of Medicine. Of these, 35 patients (2.6%) were diagnosed with severe POPF and were treated intensively. Patients underwent preoperative assessments including gastric endoscopy, computed tomography (CT) scans, and laboratory tests. Based on the preoperative diagnosis, total or distal gastrectomy and sufficient lymphadenectomy was performed, mainly according to the Japanese guidelines for the treatment of gastric cancer[13]. Patients with T1 and N0 tumors underwent D1, D1 + α, or D1 + β lymphadenectomy. Patients with T2 or more advanced tumors and those with N1 or more advanced tumors underwent D2 lymphadenectomy. Briefly, D1 lymphadenectomy indicated dissection of the perigastric lymph nodes (nodal stations No. 1, 2, 3, 4, 5 and 6) and D1 + α lymphadenectomy indicated dissection of the perigastric lymph nodes and nodes at the base of the left gastric artery (No. 7). D1 + β lymphadenectomy indicated dissection of the perigastric lymph nodes and stations No. 7, 8a (anterosuperior group of the common hepatic artery), and 9 (celiac artery) lymph nodes. In the D2 dissection, the perigastric lymph nodes and all second-tier lymph nodes were completely retrieved. Depending on the location of the tumor, lymphadenectomy was added along the distal side of the splenic artery (No. 11d) and at the splenic hilum (No. 10), together with splenectomy or splenectomy with distal pancreatectomy[14].

Definition of POPF using the ISGPF classification

POPF was retrospectively defined according to the ISGPF definition[15]: output via an operatively placed drain (or a subsequently placed percutaneous drain) of any measurable volume of drain fluid on or after postoperative day 3, with an amylase content more than 3 times higher than the upper normal serum value. We comprehensively diagnosed POPF according to not only drain amylase (D-AMY) levels, but also changes in the properties of the drain, clinical findings, laboratory data, and imaging findings such as ultrasonography (US) or CT scans. Patients who had no drains or whose drains were removed that developed postoperative fever (< 38 ℃), leukocytosis, and peripancreatic fluid collection detected on US or CT scans were also diagnosed with POPF.

POPF was graded according to the ISGPF criteria as follows: grade A had no clinical impact and required no treatment; grade B required a change in management or adjustment in the clinical pathway; and grade C required a major change in clinical management or deviation from the normal clinical pathway and required aggressive clinical intervention. Patients requiring only repositioning of their drains belonged to Grade B POPF. Patients were classified as grade C POPF if US and CT findings showed peripancreatic fluid collection and drains needed to be placed interventionally in order to improve severe clinical data and conditions. In this study, severe POPF were regarded as a clinically significant pancreatic fistula corresponding to grade B and C POPF.

Treatment strategy for POPF following gastrectomy

Patients with POPF, which is diagnosed by high D-AMY level and have no abnormal physical finding and labo-

Table 1 Characteristics of 35 patients with postoperative pancreatic fistula following gastrectomy *n* (%)

Sex	Male	30 (86)
	Female	5 (14)
Age (yr)	mean + SD	67.3 ± 9.5
BMI (kg/m^2)	mean + SD	22.1 ± 3.2
pT-stage	T1	3 (9)
	T2	11 (31)
	T3	15 (43)
	T4	6 (17)
pN-stage	N0	10 (29)
	N1	3 (9)
	N2	9 (26)
	N3	13 (36)
pStage	I	6 (17)
	II	3 (9)
	III	18 (51)
	IV	8 (23)
Gastrectomy	Distal	10 (29)
	Total or others[1]	25 (71)
Splenectomy	Presence	21 (60)
	Absence	14 (40)
Pancreaticosplenectomy	Presence	12 (34)
	Absence	23 (66)
Lymphadenectomy	< D2	8 (23)
	D2 ⩽	27 (77)
Resection status	R0	10 (29)
	R1	15 (42)
	R2	10 (29)
ISGPF classification	Grade B	17 (49)
	Grade C	18 (51)

[1]Others; proximal gastrectomy and remnant gastrectomy. ISGPF: International Study Group on Pancreatic Fistula; BMI: Body mass index.

ratory data, could be followed without any treatments. The abdominal drainage tube is normally removed after the D-AMY level has been reduced to a level lower than three times the serum AMY level. Patients with POPF, which is diagnosed by high D-AMY level and have abnormal findings such as fever, abdominal pain and other laboratory data, start to undergo intensive POPF treatments. After emergency CT examination, if the drainage tube position is good, antibiotics, octreotide acetate and total parenteral nutrition should be started. If the fluid drainage tube position is not satisfactory, an additional or alternative drainage tube can be placed into the abnormal fluid cavity by percutaneous CT or ultrasonography-guided technique. Moreover, bacterial infection of drainage fluid and/or the suspicion of it was detected following these POPF treatments. The drainage tube would be changed into an irrigation type drainage tube. Then, continuous irrigation and drainage with saline would be performed. If these series of conservative POPF treatments were not effective, open drainage and debridement for POPF abscess by laparotomy would be performed and the irrigation type drainage tube and an enteral feeding tube would be placed. After that, comprehensive POPF treatments consist of continuous irrigation drainage with saline, antibiotics, octreotide acetate and enteral nutrition.

Statistical analysis

The χ^2 test and Fisher's exact probability test were performed for categorical variables, while the Student's *t* test and Mann-Whitney *U* test for unpaired data of continuous variables were performed to compare clinicopathological characteristics between the two groups. Multivariate stepwise logistic regression analysis was performed to identify the independent risk factors associated with Grade C POPF. Multivariate odds ratio are presented with 95%CI. In all of these analyses, *P* values less than 0.05 were considered significant.

RESULTS

Clinicopathological characteristics of patients with severe POPF

Table 1 shows the characteristics of 35 patients with severe POPF. The mean patient age was 67.3 years and the male:female ratio was 6:1. More than 80% of patients were male and the incidence of patients with pT3-T4, pStage Ⅲ-Ⅳ, and D2 or more lymphadenectomy was high. Of 35 patients with severe POPF, 17 (49%) and 18 (51%) patients were classified as grade B and grade C POPF, respectively. The median intensive treatment period of POPF was 20 d. Twenty nine patients were diagnosed with POPF by their D-AMY levels and were retrospectively judged to meet the ISGPF criteria. The remaining 6 patients were diagnosed with POPF by their clinical condition, laboratory data, and CT findings because POPF was detected after drain removal.

Comparison of clinicopathologic factors and ISGPF classification in patients with severe POPF between short and long duration intensive treatments

No current standard definition of POPF reflects the duration of intensive treatments according to the severity of POPF following gastrectomy for gastric cancer. Therefore, we compared possible clinicopathologic factors and ISGPF classification between short (< 20 d) and long (⩾ 20 d) duration intensive treatments (Table 2). The cut off value of each continuous clinical data was decided by a ROC curve. As a result, the severity of POPF according to the ISGPF classification was significantly correlated with the duration of intensive POPF treatments (P = 0.035). There were no significant differences between both groups for other clinicopathologic factors, although POPF after pancreaticosplenectomy was associated with long duration intensive POPF treatments (P = 0.149). Therefore, we confirmed that the ISGPF classification reflects the duration of intensive treatments according to the severity of POPF and is a reliable classification of POPF following gastrectomy for gastric cancer.

Comparison of clinical factors between Grade B and C POPF according to the ISGPF classification

We compared several clinical factors between grade B

Table 2 Comparison of clinicopathologic factors and International Study Group on Pancreatic Fistula classification in patients with severe postoperative pancreatic fistula between short and long duration of intensive treatments *n* (%)

Variables		Intensive treatment periods for POPF		[1]*P* value
		< 20 d	20 d ≤	
Sex	Male	10 (83)	20 (87)	
	Female	2 (17)	3 (13)	0.827
Age (yr)	< 65	5 (42)	7 (30)	
	65 ≤	7 (58)	16 (70)	0.772
BMI (kg/m^2)	< 21	5 (42)	6 (27)	
	21 ≤	7 (58)	16 (73)	0.636
pT-stage	T1 T2	3 (25)	3 (13)	
	T3 T4	9 (75)	20 (87)	0.391
pN-stage	N1 N2	5 (42)	16 (70)	
	N3	7 (58)	7 (30)	0.217
pStage	Ⅰ Ⅱ	4 (33)	5 (22)	
	Ⅲ Ⅳ	8 (67)	18 (78)	0.736
Splenectomy	Presence	7 (58)	14 (61)	
	Absence	5 (42)	9 (39)	0.827
Pancreatico-splenectomy	Presence	2 (17)	10 (43)	
	Absence	10 (83)	13 (57)	0.149
Lymphadenectomy	< D2	2 (17)	6 (26)	
	D2 ≤	10 (83)	17 (74)	0.685
Resection status	R0	5 (42)	5 (22)	
	R1	4 (33)	11 (48)	
	R2	3 (25)	7 (30)	0.728
Blood loss (g)	< 1000	3 (25)	9 (39)	
	1000 ≤	9 (75)	14 (61)	0.476
Operation time (min)	< 330	6 (50)	11 (48)	
	330 ≤	6 (50)	12 (52)	0.815
Preoperative Hb (g/dL)	< 10	5 (42)	3 (13)	
	10 ≤	7 (58)	20 (87)	0.091
Preoperative Alb (g/dL)	≤ 3.5	3 (30)	6 (29)	
	3.5 <	7 (70)	15 (71)	1.000
[2]Lymphocyte counts (/mm^3)	< 850	5 (42)	18 (78)	
	850 ≤	7 (58)	5 (22)	0.073
ISGPF classification	grade B	9 (75)	8 (35)	
	grade C	3 (25)	15 (65)	0.035[3]

[1]*P* values were derived from the χ^2 or Fisher's exact test and were considered significant at < 0.05. [2]Lymphocyte counts at the diagnosis of POPF; [3]Significant values. ISGPF: International Study Group on Pancreatic Fistula; POPF: Postoperative pancreatic fistula; BMI: Body mass index.

and C POPF according to the ISGPF classification in order to detect the predictive factors of extremely severe POPF (Table 3). As a result, older patients (P = 0.035, ≤ 65 years old *vs* < 65 years old) and those with low lymphocyte counts at the diagnosis of POPF (P = 0.007, < 1400/mm^3 *vs* ≥ 1400/mm^3) were significantly associated with Grade C POPF. The cut-off value of 1400/mm^3 is calculated by the ROC curve to distinguish between grade B and C POPF (Figure 1). The incidence of other clinical factors, which were presented in Table 3 and others such as underlying disease, methods of reconstruction, HbA1c, postoperative Hb, Alb, preoperative serum total protein, total cholesterol, triglyceride, %LVC and FEV 1.0% *etc*., did not significantly differ between both groups (data not shown). Furthermore, logistic regression analysis revealed that a low lymphocyte count was an independent risk factor by multivariate analysis [P = 0.045, OR = 10.45 (95%CI: 1.050-104.1)] (Table 4).

DISCUSSION

Until recently, there has been no universally recognized definition of POPF following gastrectomy for gastric cancer. Accordingly, different definitions of POPF have been reported, which has resulted in highly variable rates of POPF, ranging from 5.8% to 49.7%[7,10,16-20]. Therefore, it is impossible to accurately evaluate the incidence and severity of POPF. Obama *et al*[21] were the first to utilize the ISGPF classification, which was formulated as an objective definition of POPF following pancreatic surgery in 2005[15], to evaluate the feasibility of laparoscopic gastrectomy with radical lymphadenectomy for gastric cancer. The incidence of ISGPF grade B or C including both open and laparoscopic gastrectomy was 5.1% (12/233)[21]. Miki *et al*[22] also reported using the ISGPF classification that a high content of drain AMY on 1POD could be used to predict severe POPF. The incidence of ISGPF grade B or C following total gastrectomy with D2 lymphadenectomy was 22.1% (23/104). Jiang *et al*[11] recently reported that severe POPF, defined as ISGPF grade B or C, was associated with being male and a high BMI in patients undergoing laparoscopic gastrectomy for gastric cancer. The incidence of ISGPF grade B or C following laparoscopic distal gastrectomy for early gastric cancer was 4.2% (34/798). Miyai *et al*[23] advocated that simple predictive scoring system might be useful for many clinicians to assess the risk of POPF after laparoscopic gastrectomy (LAG). The incidence of ISGPF grade B or C following LAG was 3.9% (11/277). These reports clarified the significance of using the same definition of POPF and detecting the risk factors of POPF using the ISGPF classification. However, it remains unclear whether the ISGPF classification following pancreatic surgery can be applied to POPF following gastrectomy to reflect the extent of the severity of POPF and treatment outcomes.

In order to elucidate whether the ISGPF classification can be translated into a clinically useful definition for POPF following gastrectomy, we showed that differences in the severity of POPF defined by the ISGPF classification indeed reflected intensive treatment periods. As a result, we confirmed that the ISGPF classification was a reliable classification that was significantly correlated with the duration of intensive treatments in patients with severe POPF following gastrectomy for gastric cancer (Table 1). These results contribute to universal recognition of the ISGPF classification as one of the candidate definitions of POPF following gastrectomy for gastric cancer.

Although several possible risk factors associated with the occurrence of POPF have been reported, there have been no generally accepted risk factors to predict extremely severe POPF, which requires several intensive treatments. During intensive treatments, indicators that provide an objective description of the patient's condi-

Table 3 Comparison of clinical factors between grade B and C postoperative pancreatic fistula according to International Study Group on Pancreatic Fistula classification *n* (%)

Variables		Total	ISGPF classification		[1]*P* value
			Grade B (*n* = 17)	Grade C (*n* = 18)	
Sex	Male	30	14 (82)	16 (89)	
	Female	5	3 (18)	2 (11)	0.944
Age (yr)	< 65	12	9 (53)	3 (17)	
	65 ⩽	23	8 (47)	15 (83)	0.035[3]
BMI (kg/m^2)	< 21	13	9 (53)	4 (24)	
	21 ⩽	21	8 (47)	13 (76)	0.158
pT-stage	T1 T2	6	4 (24)	2 (11)	
	T3 T4	29	13 (76)	16 (89)	0.402
pN-stage	N1 N2	21	9 (53)	12 (67)	
	N3	14	8 (47)	6 (33)	0.629
pStage	Ⅰ Ⅱ	9	5 (29)	4 (22)	
	Ⅲ Ⅳ	26	12 (71)	14 (78)	0.921
Splenectomy	Presence	21	11 (65)	10 (56)	
	Absence	14	6 (35)	8 (44)	0.836
Pancreaticosplenectomy	Presence	12	4 (24)	8 (44)	
	Absence	23	13 (76)	10 (56)	0.344
Lymphadenectomy	< D2	8	3 (18)	5 (28)	
	D2 ⩽	27	14 (82)	13 (72)	0.691
Resection status	R0	10	4 (24)	6 (33)	
	R1	15	7(41)	8 (45)	
	R2	10	6 (35)	4 (22)	0.892
Blood loss (g)	< 1000	23	11 (65)	12 (67)	
	1000 ⩽	12	6 (35)	6 (33)	0.815
Operation time (min)	< 330	19	10 (59)	9 (50)	
	330 ⩽	16	7 (41)	9 (50)	0.854
Preoperative Hb (g/dL)	<10	8	5 (29)	3 (17)	
	10 ⩽	27	12 (71)	15 (83)	0.443
Preoperative Alb (g/dL)	⩽ 3.5	9	4 (27)	5 (29)	
	3.5 <	23	11 (74)	12 (71)	0.825
[2]Lymphocyte counts (/mm^3)	< 1400	26	9 (53)	17 (94)	
	1400 ⩽	9	8 (47)	1 (6)	0.007[3]

[1]*P* values were derived from the χ^2 or Fisher's exact test and were considered significant at < 0.05; [2]Lymphocyte counts at the diagnosis of postoperative pancreatic fistula; [3]Significant values. ISGPF: International Study Group on Pancreatic Fistula; BMI: Body mass index.

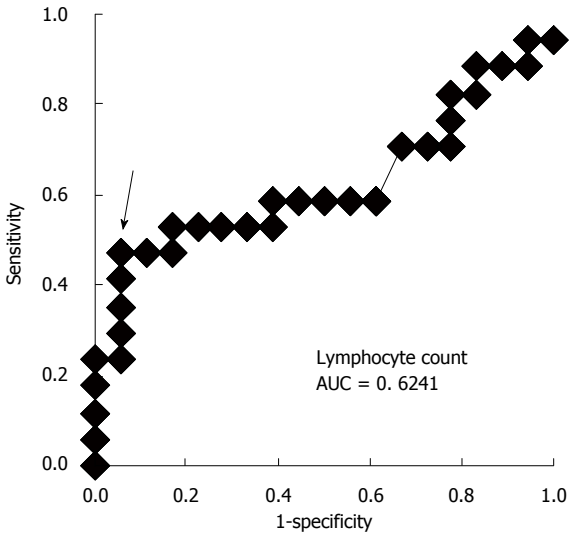

Figure 1 The cut-off value of 1400/mm^3 is calculated by the receiver operating characteristic-curve to distinguish between grade B and C postoperative pancreatic fistula. Arrow is the point of cut-off value.

tion at the diagnosis of POFP are useful for understanding the complications that may be encountered. In this study, we demonstrated that older patients (P = 0.035) and those with lower lymphocyte counts at the diagnosis of POPF (P = 0.007) were significantly associated with extremely severe grade C POPF, and a low lymphocyte count was identified as an independent risk factor by multivariate analysis (P = 0.045, OR = 10.45) (Table 2).

Table 4 Results of multivariable logistic regression; risk factor for extremely severe postoperative pancreatic fistula

Covariate	OR	95% Confidence limit	*P* value
Lymphocyte counts (/mm^3)			
< 1400 *vs* 1400 ⩽	10.45	1.05-104.1	0.045
Age (yr)			
65 ⩽ *vs* < 65	3.39	0.602-1.886	0.166

At first, we hypothesized that there were some differences among the previously reported risk factors associated with the occurrence of POPF between grade B and C in patients with severe POPF. However, contrary to our expectations, there was no correlation with the previously reported factors associated with the occurrence of POPF such as radical or extended lymphadenectomy[3,4], splenectomy or pancreaticosplenectomy[5-7], a higher BMI

and VFA, being male, hyperlipidemia, and comorbidities[8-11]. In this study, blood lymphocyte counts at the diagnosis of POPF were the only independent risk factor to predict the severity of POPF patients. This result implies that factors associated with the occurrence of POPF may not affect the severity of severe POPF by changing it from grade B to C POPF.

The reason why a low lymphocyte count was the only independent risk factor to predict extremely severe POPF remains unclear. One possible reason is that a change in the severity of POPF from grade B to C POPF may be associated with host-related immunity. Hogan *et al*[24] suggested that a perioperative reduction in circulating lymphocyte levels was an independent predictive factor for wound complications following excisional breast cancer surgery. As discussed in their report, which resulted in selective antibiotic prophylaxis being required for these immune-compromised patients, POPF patients with lower lymphocyte counts at diagnosis may require more intensive treatments to avoid POPF developing into extremely severe POPF, such as grade C POPF. Another possible reason was that a low lymphocyte count may be caused by a delay in the diagnosis of severe POPF and this data may reflect a pre-septic state[25,26]. Patients with POPF, which have only abnormal D-AMY data, can be followed without any treatment. These patients mainly resulted in grade A POPF; however, some of these patients may later develop severe POPF. Indeed, in our study, severe POPF patients with grade B started intensive treatments after an average of 5.7 d. In contrast, extremely severe POPF patients with grade C started these treatments after an average of 10.3 d (data not shown). Severe POPF sometimes presents no clinical symptoms such as high grade fever and abdominal pain until some time later. However, surgeons should bear in mind that some POPF patients may develop extremely severe POPF. Therefore, a lower lymphocyte count could provide an objective description and is useful for understanding the complications that may be encountered.

In conclusion, we confirmed that the ISGPF classification of POPF following gastrectomy was a reliable classification that correlated with the duration of intensive treatments in patients with severe POPF. Furthermore, we clarified an independent risk factor to predict the worst outcome of POPF treatment with special reference to the ISGPF classification. Therefore, caution and intensive care are required for older POPF patients and those with lower lymphocyte counts at the diagnosis of POPF.

COMMENTS

Background

Despite recent advances in less invasive treatment techniques and the perioperative management of gastric cancer, postoperative pancreatic fistula (POPF) is still a major complication following gastrectomy. Once POPF develops, it sometimes contributes to lethal complications, such as abdominal abscesses, secondary anastomotic leakage, and intra-abdominal hemorrhage. However, to date, after patients develop POPF, there are no generally accepted risk factors to predict these patients to change severe POPF.

Research frontiers

Indicators that provide an objective description of the patient's condition at specific points in the disease process of POPF are useful to improve understanding of the complications that may be encountered. In this study, the authors confirmed that the severity of POPF according to the International Study Group on Pancreatic Fistula (ISGPF) classification was correlated with the duration of intensive POPF treatments. Furthermore, they clarified an independent risk factor to predict the worst outcome of POPF treatment with special reference to the ISGPF classification.

Innovations and breakthroughs

Between 1997 and 2010, 1341 consecutive patients underwent gastrectomy for gastric cancer. Of these, 35 patients (2.6%) were diagnosed with Grade B or C POPF according to the ISGPF classification and were treated intensively. The severity of POPF according to the ISGPF classification was significantly correlated with the duration of intensive POPF treatments ($P = 0.035$). Regarding the clinical factors to distinguish extremely severe POPF, older patients ($P = 0.035$, $\geqslant$ 65 years old *vs* < 65 years old) and those with lower lymphocyte counts at the diagnosis of POPF ($P = 0.007$, < 1400/mm^3 *vs* $\geqslant$ 1400/mm^3) were significantly correlated with Grade C POPF, and a low lymphocyte count was an independent risk factor by multivariate analysis [$P = 0.045$, OR = 10.45 (95%CI: 1.050-104.1)].

Applications

The ISGPF classification of POPF following gastrectomy was a reliable classification that correlated with the duration of intensive treatments in patients with severe POPF. Caution and intensive care are required for older POPF patients and those with lower lymphocyte counts at the diagnosis of POPF.

Terminology

POPF: POPF following gastrectomy for gastric cancer is still a major complication following gastrectomy. ISGPF: grade A had no clinical impact and required no treatment; grade B required a change in management or adjustment in the clinical pathway; and grade C required a major change in clinical management or deviation from the normal clinical pathway and required aggressive clinical intervention.

Peer review

This is a good descriptive study showing that the ISGPF classification was reliable in patients with POPF following gastrectomy for gastric cancer. Caution and intensive care are required for older POPF patients and those with lower lymphocyte counts at the diagnosis of POPF.

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BRIEF ARTICLE

Long-term follow up of endoscopic resection for type 3 gastric NET

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Abstract

AIM: To clarify the short and long-term results and to prove the usefulness of endoscopic resection in type 3 gastric neuroendocrine tumors (NETs).

METHODS: Of the 119 type 3 gastric NETs diagnosed from January 1996 to September 2011, 50 patients treated with endoscopic resection were enrolled in this study. For endoscopic resection, endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD) was used. Therapeutic efficacy, complications, and follow-up results were evaluated retrospectively.

RESULTS: EMR was performed in 41 cases and ESD in 9 cases. Pathologically complete resection was performed in 40 cases (80.0%) and incomplete resection specimens were observed in 10 cases (7 *vs* 3 patients in the EMR *vs* ESD group, $P = 0.249$). Upon analysis of the incomplete resection group, lateral or vertical margin invasion was found in six cases (14.6%) in the EMR group and in one case in the ESD group (11.1%). Lymphovascular invasions were observed in two cases (22.2%) in the ESD group and in one case (2.4%) in the EMR group ($P = 0.080$). During the follow-up period (43.73; 13-60 mo), there was no evidence of tumor recurrence in either the pathologically complete resection group or the incomplete resection group. No recurrence was reported during follow-up. In addition, no mortality was reported in either the complete resection group or the incomplete resection group for the duration of the follow-up period.

CONCLUSION: Less than 2 cm sized confined submucosal layer type 3 gastric NET with no evidence of lymphovascular invasion, endoscopic treatment could be considered at initial treatment.

Key words: Stomach; Neuroendocrine tumor; Endoscopic resection; Treatment; Carcinoid

Core tip: Endoscopic treatment was suitable for tumors measuring approximately 20 mm or smaller in size, with no lymph node or distant metastasis and limited to the submucosal layer of type 3 gastric neuroendocrine tumors (NETs), similar to endoscopic treatment guidelines applied to other gastrointestinal NETs.

Kwon YH, Jeon SW, Kim GH, Kim JI, Chung IK, Jee SR, Kim HU, Seo GS, Baik GH, Choi KD, Moon JS. Long-term follow up of endoscopic resection for type 3 gastric NET. *World J Gastroenterol* 2013; 19(46): 8703-8708 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i46/8703.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i46.8703

INTRODUCTION

Neuroendocrine tumors (NETs) are slow-growing malignancies with distinct biological and clinical characteristics. Although these tumors have long been a source of clinical and pathologic interest, their fundamental biology still eludes precise delineation[1]. Despite the relative rarity of gastric NETs, their diagnosis is increasing due to the recent widespread use of diagnostic endoscopy[2-4]. Yearly age-adjusted incidence is approximately 0.2 per population of 100000.

Enterochromaffin-like (ECL) cells, the main endocrine cell types in type 1 and type 2 gastric NETs, are highly susceptible to gastrin trophic stimuli. Under circumstances that cause hypergastrinemia, such as chronic atrophic gastritis (CAG) in pernicious anemia (type 1) or gastrin-producing neoplasms in Zollinger-Ellison syndrome (ZES)/multiple endocrine neoplasia (MEN) 1 (type 2), multiple ECL cell carcinoids occur in the oxyntic corpus and fundus mucosa of the stomach[5,6]. Type 1 and 2 gastric NETs are usually considered benign, with a low risk of malignancy. However, type 3 gastric NETs are composed of different endocrine cells, which grow sporadically, irrespective of gastrin, in an otherwise normal mucosa. Most of these tumors show lymphoinvasion, angioinvasion, and deep wall invasion at the time of diagnosis, and they often present with metastases, which are found in 50%-70% of well-differentiated, and in up to 100% of poorly differentiated tumors[6-9]. As a worse overall mortality of type 3 gastric NETs, aggressive surgery is considered the initial therapeutic approach, generally. Many reports on the efficacy of endoscopic treatment for gastric NETs have been published[10-13]. However, few studies have reported on endoscopic treatment of type 3 gastric NETs.

In this study, we will conduct a retrospective review of the outcomes and long-term prognosis of endoscopic treatment on type 3 gastric NETs. In addition, we demonstrate the efficacy of endoscopic treatment on type 3 gastric NETs.

MATERIALS AND METHODS

Ethics

This work has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. This study was approved ethically by University Hospital Kyungpook Trust (KNUMC_ 12-1005). All patients provided informed written consent for this study.

Patients

After receiving appropriate Institutional Review Board approval, members of the Korean college of Helicobacter and Upper Gastrointestinal Research retrospectively enrolled patients who were diagnosed with histologically proven gastric NETs from 10 hospitals between January 1996 and September 2011. Based on endoscopic findings, all gastric NETs were classified according to the Paris endoscopic classification[14]. Abdominal computed tomography (CT) scans were available for diagnosis of lymph node involvement or other organ metastasis. These patients were then analyzed with respect to their presenting signs and symptoms, associated disease, tumor characteristics (number, size, site, and the presence of metastasis), and outcome. From the 225 gastric NETs, we reviewed patients' plasma gastrin levels and other associated diseases, such as ZES and multiple endocrine neoplasia (MEN) type 1, to diagnose type 3 gastric NETs. The exact criteria used to decide between endoscopic or surgical treatment was dependent on the tumor size, tumor shape (combined ulceration or depressed lesions), or evidence of adjacent lymph node metastasis.

Histopathologic findings and TNM stage of gastric NETs

Resection specimens processed by formalin fixation were serially sectioned at 2 mm intervals, and tumor involvement to the lateral and vertical margins was assessed. In addition, histopathological type, tumor size, depth of invasion, and lymphovascular invasion were evaluated microscopically. Pathologically complete resection was defined according to the following findings: (1) en bloc resection; (2) the tumor was a well-differentiated neuroendocrine tumor (classical-type carcinoid) according to World Health Organization (WHO) classification[15]; (3) tumor invasion was limited to the submucosal layer; (4) no lateral and vertical margin involvement; and (5) no lymphovascular invasion.

Endoscopic findings and endoscopic mucosal resection and endoscopic submucosal dissection procedures

We evaluated tumor characteristics, such as the measured size, number, and location of tumors. Tumor size was estimated using biopsy forceps (FB 21K-1; Olympus Medical Systems Co, Tokyo, Japan), which was approximately 6 mm in length when opened. Tumor location was reported according to the longitudinal axis (fundus, cardia, body, or antrum). All lesions were imaged with adjacent anatomical structures to ensure that the exact location of

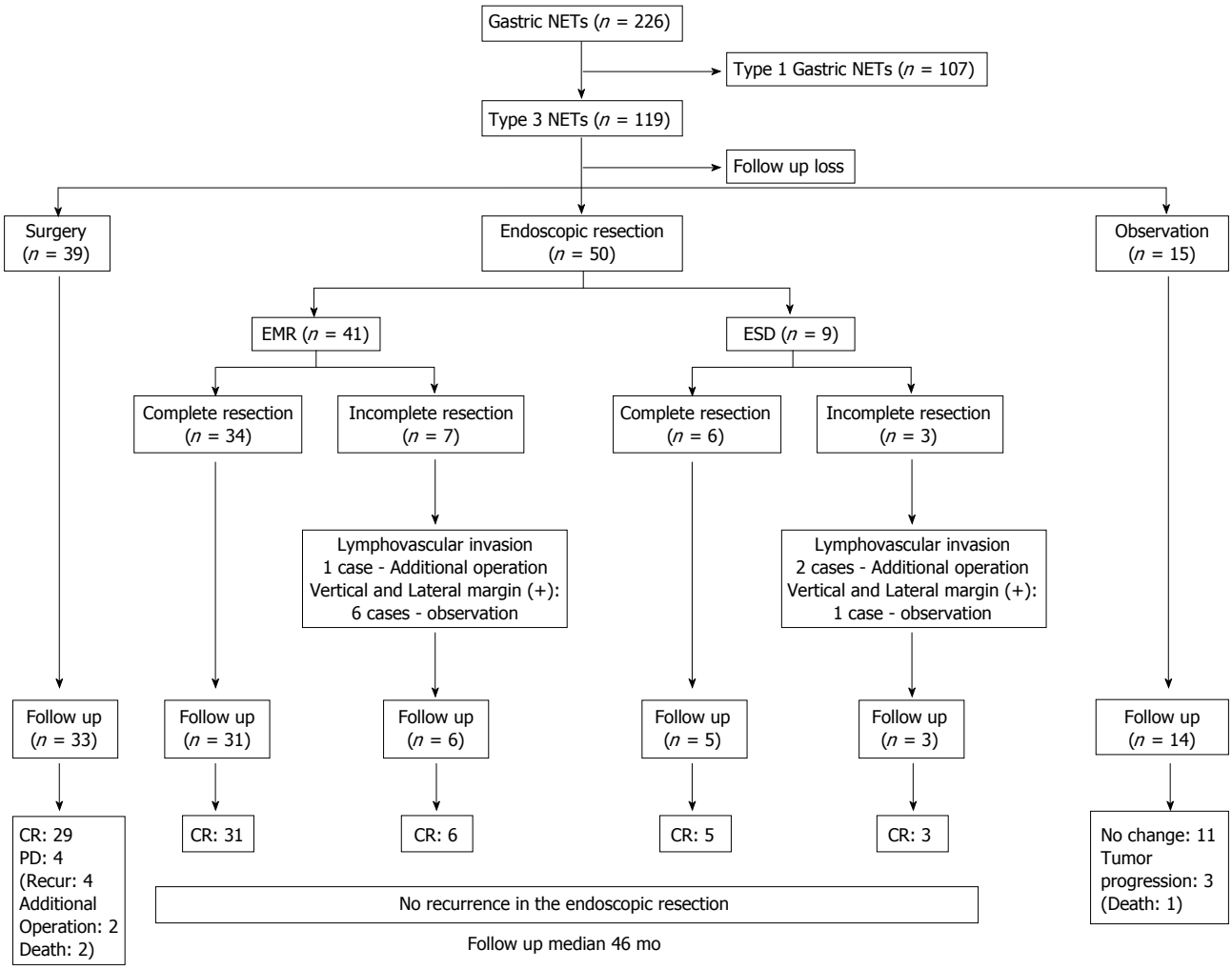

Figure 1 Flow chart of type 3 gastric neuroendocrine tumors. Of all type 3 gastric neuroendocrine tumors (NETs) (*n* = 119), 39 patients were treated with surgery, 50 patients were treated using an endoscopic method, and 15 patients were followed up only by observation. In the endoscopic treatment group, 41 patients were treated with endoscopic mucosal resection (EMR), and nine patients were treated with endoscopic submucosal dissection (ESD). Upon analysis of the resected specimens, histologically incomplete resections were found in seven cases in the EMR group and three cases in the ESD group, and lymphovascular invasion was found in one case in the EMR group and two cases in the ESD group. All cases of lymphovascular invasion were treated with an additional operation. During the median follow-up duration (46 mo), there was no recurrence of gastric NETs in the endoscopic resection group.

the tumor was recorded and proved histologically by endoscopic biopsy. Endoscopic ultrasonography was used for measuring the depth of invasion of gastric NETs. Endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD) was performed after obtaining informed consent. Submucosal injection of saline mixed with epinephrine was performed to elevate tumor tissues from the underlying muscularis propria. Next, EMR, using a hood and snare, or submucosal dissection was applied for removal of the lesion.

Follow-up after endoscopic resection

The follow-up program consisted of endoscopic examinations at three, six, and twelve-month intervals, and CT scans and blood tests were performed at 12-month intervals. Follow-up endoscopy was performed depending on the follow-up program, and for histological examinations of NETs recurrence, biopsies were performed at iatrogenic ulcer scar lesions that had undergone endoscopic treatment. CT examination findings were normal in all patients at the end of follow up.

Statistical analysis

All continuous variable data are presented as the mean ± SE. Statistical significance was calculated using unpaired Student's *t* test. To assess the difference between two procedures, univariate analysis was performed using Student's *t*-test. Statistical significance was set at 0.05. All analyses were performed using SPSS version 18.0 (SPSS Inc., United States).

RESULTS

Patient baseline characteristics

Overall, in the 226 cases of gastric NETs, 119 cases (52.4%) were diagnosed as type 3 gastric NETs. Of the 119 patients, 50 patients (42.0%) received endoscopic interventions for the treatment of type 3 gastric NET lesions (Figure 1). The average age of the patients was 58.6 (25-85) years. Twenty-eight (56.0%) patients were

Table 1 Patient characteristics of 51 gastric endocrine tumors who underwent endoscopic resection *n* (%)

Male:female	28:22
Mean age, yr	58.6 ± 12.2
Associated symptoms	
Abdominal discomfort	14 (28.0)
Body weight loss	1 (2.0)
Diarrhea	1 (2.0)
Other symptom	1 (2.0)
Associated disease	
Diabetes mellitus	5 (10.0)
Thyroid disease	1 (2.0)
Combined other malignancy	2 (4.0)
Number of tumors	
1	48 (96.0)
≥ 2	2 (4.0)
Tumor location	
Antrum	4 (8.0)
Body	38 (76.0)
Fundus or cardia	8 (16.0)
Tumor size	
≤ 10 mm	33 (66.0)
> 10 mm	17 (34.0)
EUS invasion depth	
Mucosa and submucosa	49 (98.0)
MP	1 (2.0)
Treatment methods	
EMR	41 (82.0)
ESD	9 (18.0)

GET: Gastric endocrine tumor; MP: Muscularis propria; EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection.

male and 22 (44.0%) patients were female. Asymptomatic patients were the most common, and abdominal discomfort was the second most common presenting symptom (28.0%) in patients who had type 3 gastric NETs. Upon analysis of the associated underlying disease, five patients (10.0%) had diabetes mellitus (DM), one patient (2.0%) had thyroid disease and early gastric cancer (EGC), and two patients (4.0%) had other combined malignancies (Table 1).

Tumor characteristic and metastasis

Based on the endoscopic findings, superficial elevated type (type IIa) and solitary lesions (96%) were most prevalent. Upon analysis of the location of the type 3 gastric NETs, 38 lesions (76.0%) were found on the body. Based on the EUS evaluation, there were 49 cases (98.0%) of confined tumors in the mucosal or submucosal layer, and one tumor (2.0%) was suspicious of invasion into the muscular propria (MP) layer. No lymphatic invasion or other organ metastasis findings was observed in the imaging stud (Table 1).

Treatment modality and results

Of the 50 patients who had been treated with endoscopic intervention, 41 patients (82.0%) were treated by EMR and 9 patients (18.0%) were treated by ESD. The mean tumor size of the gastric NETs was 10.2 ± 6.3 mm, and compared with the mean tumor size, no significant difference was observed between the two groups (9.3 mm *vs* 14.2 mm in the EMR *vs* ESD group, $P = 0.055$). All tumors were determined as pathologically well-differentiated neuroendocrine tumors. Upon analysis of the resected specimens, 11 tumors and six tumors in the EMR and ESD groups, respectively, were gastric NETs measuring 10 mm or more in size ($P = 0.031$); pathologically complete resections were achieved in 40 cases (80.0%), and incomplete resection specimens were seen in 10 cases (7 *vs* 3 patients in the EMR *vs* ESD group, $P = 0.249$). Lateral or vertical margin invasion was found in six cases (14.6%) in the EMR group and in one case in the ESD group (11.1%). Lymphovascular invasions were observed in two cases (22.2%) in the ESD group and in one case (2.4%) in the EMR group ($P = 0.080$) (Table 2).

Table 2 Treatment outcomes after endoscopic treatment of gastric endocrine tumors *n* (%)

	EMR (*n* = 41)	ESD (*n* = 9)	*P* value
Mean resection size (range, mm)	9.3 ± 5.6	14.2 ± 7.8	0.055
Tumor size > 10 mm	11 (26.8)	6 (66.7)	0.031
Pathologically complete resection	35 (85.4)	6 (66.7)	0.249
Lymphovascular invasion	1 (2.4)	2 (22.2)	0.080
Additional operation	1 (2.4)	2 (22.2)	0.080

EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection.

The mean tumor size of a complete resection was 9.6 (2-32) mm, and the size for an incomplete resection was 12.4 (3-20) mm ($P = 0.011$). The mean tumor size of lymphovascular invasion cases was larger than that of the no lymphovascular invasion group, however, there was no significant difference ($P = 0.416$) (Table 3). All cases of with a lymphovascular invasion tumor underwent an additional operation, while other incomplete resection cases were followed up by observation (Figure 1). There were no complications after the endoscopic treatment procedures.

Follow-up

Of the 50 patients who underwent endoscopic treatment, five patients (10.0%) were lost to follow-up, and 45 patients (90%) were included in the follow-up. The median follow-up duration was 46 (13-60) mo. No evidence of tumor recurrence was found upon endoscopic and histological examinations in both groups. There was also no evidence of recurrence during follow-up imaging studies. In addition, no mortality was reported in either the complete resection group or the incomplete resection group during the follow-up duration. If 5 years was used as a cut-off point, 20 patients showed a disease-free state during this period.

DISCUSSION

Carcinoids were first described by Oberndorfer in 1907 to describe a group of tumors of the gastrointestinal tract that had a relatively indolent course and were con-

Table 3 Analysis of resected tumors

	Tumor size	*P* value
Complete resection (range, mm)		
Yes (n = 40)	9.6 ± 6.3	0.011
No (n = 10)	12.4 ± 6.1	
Lymphovascular invasion (range, mm)		
Yes (n = 3)	16.3 ± 4.2	0.416
No (n = 47)	9.8 ± 6.2	

sidered to be intermediate between adenomas and carcinomas in terms of malignancy potential. Currently, these tumors are also known by the modern term of gastric NETs, which include a subset of tumors demonstrating features of neuroendocrine differentiation[15]. Surgery has been the most common treatment of gastric NETs; however, these tumors often receive suboptimal management, and some patients still undergo inappropriate surgery. As the diagnosis of gastric NETs is increasing with the widespread use of screening diagnostic endoscopy, treatment using the endoscopic method is becoming a matter of concern. In type 1 gastric NETs, endoscopic polypectomy or endoscopic mucosal resection is small (< 1 cm) and few (< 3-5 cm) in number[16] because the clinical behavior of these tumors is usually indolent. Most are grade 1 tumors with TNM stage I disease and no mortality during prolonged follow-up[17]. Type 3 gastric NETs represent 15%-25% of NETs and are not related to hypergastrinemia and ECL hyperplasia. The lesions are typically solitary, larger than 1-2 cm, ulcerated, and deeply invasive. The lesions are usually located in the gastric fundus and body, but may also occur in the antrum; they are also more frequent in males[6,18-20] and are characterized by a far more aggressive course. Type 3 gastric NETs present with lymph node and distant metastases in more than 50% of cases. Therefore, partial or total gastrectomy with local lymph node resection is considered an acceptable treatment[21,22] in the absence of visceral metastases. Additionally, systemic chemotherapy is also considered appropriate if surgery is not feasible, even if, thus far, the results are not very encouraging[23]. Only small (< 10 mm), well differentiated (G1) type 3 gastric NETs may be treated non-operatively by endoscopic resection. Because of the generally favorable tumor biology, surgery and/or local ablation should be considered even in metastatic gastric NETs[3]. Recently, Saund *et al*[24] reported that tumor size and depth can predict lymph node metastasis for gastric NETs and that endoscopic resection may be appropriate for intraepithelial (IE) tumors <2 cm and perhaps tumors < 1 cm invading into the lamina propria or submucosa. In our present study, complete pathological resections were achieved in 80.4% of patients (85.4% in the EMR group *vs* 66.7% in the ESD group). Better results for the pathological complete resection rate for treatment have usually been reported with the ESD technique. However, in the current study, the EMR group showed a more preferable complete resection rate compared with the ESD group. We presumed that tumor size is a contributing factor. Based on analysis of resected tumor size, the mean tumor size of the ESD group was larger than that of the EMR group (P = 0.055), and the pathologically complete resection ratio showed no significant difference in both modality groups (P = 0.249). Even in cases with tumor sizes greater than 10 mm (14 cases), which were confined to the submucosal layer and no lymphovascular invasion, endoscopic treatment showed no recurrence during the follow-up duration. Considering these factors, the ESD technique was useful for large type 3 gastric NETs. The long-term results of the endoscopic treatment only group (n = 43) showed no recurrence or mortality. Therefore, we could conclude that endoscopic treatment was suitable for tumors measuring approximately 20 mm or smaller in size, with no lymph node or distant metastasis and limited to the submucosal layer of type 3 gastric NETs, similar to endoscopic treatment guidelines applied to other gastrointestinal NETs.

Our study has some limitations. First, this study is a retrospective analysis of clinical records. However, the data are believed to be reliable because all patients with type 3 gastric NETs treated using the endoscopic method at 10 institutions between January 1996 and September 2011 were included. The second limitation is that this study has a possible selection bias because it was not randomized. However, we consider the selection bias to be minimal because the patient characteristics and the median tumor sizes of patients with type 3 gastric NETs were not different. Third, the outcome of the endoscopic resection and selection of methods for endoscopic resection were different for each institution. However, each operator had sufficient skill to perform the endoscopic procedure, and the modality of endoscopic treatment was generally accepted for the treatment of gastric NETs. The final limitation is that we enrolled patients according to the WHO 2000 system for NET classification, due to retrospective study design. Therefore, we could not evaluate tumor histology on the basis of proliferative activity (Ki-67 index, mitotic rate) in which gastric NETs are graded as G1, G2, or G3.

In a conclusion, if the tumor is confined in the submucosal layer, there is no evidence of lymphovascular invasion, and the tumor size is smaller than 2 cm, endoscopic treatment could be applied for the initial treatment of type 3 gastric NETs.

COMMENTS

Background

Lots of controversies still exist about the optimal treatment of gastric neuroendocrine tumors (NETs). Type 3 gastric NETs are known as more aggressive disease course compared with type 1 gastric NETs. So, management of type 3 gastric NETs are comparable to that used for gastric adenocarcinomas, which includes partial or total gastrectomy with extended lymph node resection. However, in the case of small sized tumor, endoscopic resection is applied for initial treatment, nowadays.

Research frontiers

To evaluate of the long-term results and to prove the usefulness of endoscopic resection in type 3 gastric NETs.

Innovations and breakthroughs

Endoscopic treatment was suitable for tumors measuring approximately 20 mm or smaller in size, with no lymph node or distant metastasis and limited to the submucosal layer of type 3 gastric NETs.

Applications

This present study suggest that the tumor size, the depth of invasion and evidence of lymphovascular invasion must be considered before performing endoscopic treatment for type 3 gastric NETs.

Peer review

This study described the efficacy of endoscopic resection for the type 3 gastric NETs which size is less than 2 cm, confined submucosal layer, and no evidence of lymphovascular invasion.

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BRIEF ARTICLE

Pattern and distribution of colonic diverticulosis: Analysis of 2877 barium enemas in Thailand

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Abstract

AIM: To determine the pattern and distribution of colonic diverticulosis in Thai adults.

METHODS: A review of the computerized radiology database for double contrast barium enema (DCBE) in Thai adults was performed at the Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. Incomplete studies and DCBE examinations performed in non-Thai individuals were excluded. The pattern and distribution of colonic diverticulosis detected during DCBE studies from June 2009 to October 2011 were determined. The occurrence of solitary cecal diverticulum, rectal diverticulum and giant diverticulum were reported. Factors influencing the presence of colonic diverticulosis were evaluated.

RESULTS: A total of 2877 suitable DCBE examinations were retrospectively reviewed. The mean age of patients was 59.8 ± 14.7 years. Of these patients, 1778 (61.8%) were female and 700 (24.3%) were asymptomatic. Colonic diverticulosis was identified in 820 patients (28.5%). Right-sided diverticulosis (641 cases; 22.3%) was more frequently reported than left-sided diverticulosis (383 cases; 13.3%). Pancolonic diverticulosis was found in 98 cases (3.4%). The occurrence of solitary cecal diverticulum, rectal diverticulum and giant diverticulum were 1.5% (42 cases), 0.4% (12 cases), and 0.03% (1 case), respectively. There was no significant difference in the overall occurrence of colonic diverticulosis between male and female patients (28.3% *vs* 28.6%, $P = 0.85$). DCBE examinations performed in patients with some gastrointestinal symptoms revealed the frequent occurrence of colonic diverticulosis compared with those performed in asymptomatic individuals (29.5% *vs* 25.3%, $P = 0.03$). Change in bowel habit was strongly associated with the presence of diverticulosis (a relative risk of 1.39; $P = 0.005$). The presence of diverticulosis was not correlated with age in symptomatic patients or asymptomatic individuals ($P > 0.05$).

CONCLUSION: Colonic diverticulosis was identified in 28.5% of DCBE examinations in Thai adults. There was no association between the presence of diverticulosis and gender or age.

Key words: Colonic diverticulosis; Diverticular disease; Barium enema; Pattern; Thailand; Cecal diverticulum; Rectal diverticulum; Giant diverticulum

Core tip: Based on this study in the largest university hospital in Thailand, colonic diverticulosis was identified in 28.5% of double contrast barium enemas performed in Thai adults. The incidence of colonic diverticulosis in the present study was markedly higher than that previously reported from hospital-based data of colonic diverticulosis in Thailand in 1980. This study also demonstrated that there was no significant association be-

tween the presence of diverticulosis and gender or age. However, colonic diverticulosis was more commonly reported in patients with some gastrointestinal symptoms, especially those with a change in bowel habit.

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INTRODUCTION

Colonic diverticulosis is a common gastrointestinal condition in which the large intestine contains outpouchings of the mucosa and submucosa through a weak area of the colon[1]. However, the actual prevalence of colonic diverticulosis is difficult to determine because most people with colonic diverticula are asymptomatic[2]. Double contrast barium enema (DCBE) is regarded as the investigation of choice for demonstrating the presence and extent of colonic diverticulosis[3,4]. It is evident that the prevalence and pattern of colonic diverticulosis differ among ethnic groups and lifestyles[5,6]; left-sided diverticulosis is most common in Western and developed countries, while right-sided diverticulosis is more prevalent in Asian and developing countries[4,7,8].

Although some data on colonic diverticulosis from Asian countries are available[6,9,10], information on colonic diverticulosis in the region of Southeast Asia is limited and outdated[11]. As the characteristics of colonic diverticulosis have changed with time[12,13], this study aimed to determine the pattern and distribution of colonic diverticulosis in Thai adults in recent years.

MATERIALS AND METHODS

After obtaining approval from our Institutional Review Board (SIRB 634/2554), a review of the computerized radiology database for DCBE in Thai adults (defined as individuals aged ⩾ 18 years) was performed at the Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. All findings of colorectal lesions detected at DCBE from June 2009 to October 2011 were analyzed. Incomplete studies, e.g. patients who were unable to hold barium or DCBE performed in patients with colonic obstruction, were excluded. Barium studies in non-Thai individuals were also excluded. Written informed consent was given by all patients before undergoing fluoroscopic DCBE. The detailed techniques and interpretation of standard fluoroscopic DCBE performed in our institute were previously reported[14]. Briefly, DCBE demonstrates a diverticulum as a barium-filled outpouching of the colon which is joined to the colonic wall by a neck. The DCBE findings were interpreted and reported by a staff gastrointestinal radiologist.

Patients' characteristics, indication for DCBE, and anatomical distribution of colonic diverticula were analyzed. In this study, the colon was divided into 3 parts: the right-sided colon (the cecum, the ascending colon, and the hepatic flexure of the colon), the transverse colon, and the left-sided colon (the splenic flexure of the colon, the descending colon, the sigmoid colon, and the rectum). Right colonic diverticulosis was defined as a diverticulum, or diverticula, detected on DCBE in the right-sided colon regardless of the involvement of the remaining colon. Left colonic diverticulosis was defined as a diverticulum, or diverticula, detected on DCBE in the left-sided colon regardless of the involvement of the remaining colon. The presence of diverticula in all three colonic segments was defined as pancolonic diverticulosis. Of note, a rectal diverticulum was defined as a diverticulum found below the imaginary line between the sacral promontory and the pubic symphysis on the lateral pelvic view of DCBE. A giant diverticulum was defined as a diverticulum demonstrated on DCBE with a diameter of ⩾ 4 cm.

Statistical analysis

All data were prepared and compiled using the Statistical Package for the Social Sciences program version 11.3 for Windows (SPSS Inc, Chicago, IL, United States). The prevalence and distribution of colonic diverticulosis detected at DCBE were analyzed with 95%CI analysis for Windows (Statistics with Confidence, 2nd Edition, BMJ Books, London 2000). The Mann-Whitney *U* test was used to compare the prevalence of diverticulosis between gender, and between symptomatic patients and asymptomatic individuals. Of note, asymptomatic individuals were defined as those without any gastrointestinal tract symptoms. The correlation between age and the presence of colonic diverticular disease was analyzed using a regression analysis. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

A total of 2877 suitable DCBE examinations were retrospectively reviewed. The mean age of patients was 59.8 ± 14.7 years (range 18-100 years). Of these patients, 1778 (61.8%) were female and 700 (24.3%) were asymptomatic. Colonic diverticulosis was identified on DCBE in 820 patients (28.5%). Right-sided diverticulosis (641 cases; 22.3%) was more frequently found than left-sided diverticulosis (383 cases; 13.3%). Pancolonic diverticulosis and solitary cecal diverticulum were found in 98 cases (3.4%) and 42 cases (1.5%), respectively (Table 1). Rectal diverticulum was seen in 12 cases (0.4%), and it was exclusively associated with the presence of sigmoid diverticulosis. A giant sigmoid diverticulum was demonstrated on DCBE in one case (0.03%). Figure 1 shows the distribution of diverticulosis stratified by colonic segment. Besides colonic diverticula, other major findings included 25 advanced adenomas (0.87%), 76 colorectal cancers (2.64%; 18 in the right-sided colon, 28 in the left-

Table 1 Percentage and distribution of colonic diverticulosis by location (from total number of 2877 double contrast barium enemas studied)

Location	No. of cases	Percentage of total 820 colonic diverticulosis	Percentage of total 2877 DCBEs studied (95%CI)
Right-sided only[1]	383	46.7	13.3 (12.1-14.6)
Left-sided only	153	18.7	5.3 (4.6-6.2)
Transverse only	10	1.2	0.3 (0.2-0.6)
Extended right-sided (right + transverse)	44	5.4	1.5 (1.1-2.0)
Extended left-sided (left + transverse)	16	2.0	0.6 (0.3-0.9)
Bilateral (right + left)	116	14.1	4.0 (3.4-4.8)
Pancolonic (right + transverse + left)	98	12.0	3.4 (2.8-4.1)
Total	820	100	28.5 (26.9-30.2)
Right colonic diverticulosis	641	78.2	22.3 (20.8-23.8)
Left colonic diverticulosis	383	46.7	13.3 (12.1-14.6)
Transverse colonic diverticulosis	168	20.5	5.8 (5.0-6.8)

[1]Right-sided only diverticulosis included 42 cases of solitary cecal diverticulum. DCBE: Double contrast barium enema.

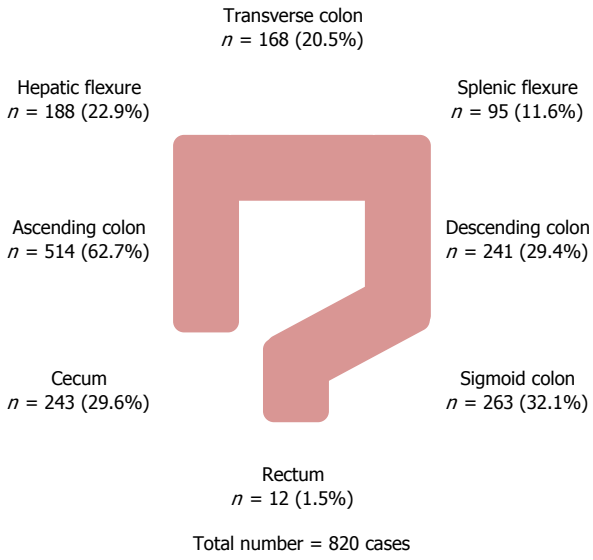

Figure 1 Distribution of colonic diverticulosis stratified by colonic segment (total number of colonic diverticulosis = 820 cases).

sided colon and 30 in the rectum), and 4 ileocecal Crohn's disease (0.14%).

There was no significant difference in the occurrence of colonic diverticulosis between male and female patients (28.3% *vs* 28.6%, $P = 0.85$). However, DCBE examinations performed in patients with some gastrointestinal symptoms revealed the frequent occurrence of colonic diverticulosis compared with those performed in asymptomatic individuals (29.5% *vs* 25.3%; $P = 0.03$). Change in bowel habit was strongly associated with the presence of diverticulosis (RR = 1.39, 95%CI: 1.14-1.70, $P = 0.005$), whereas patients with abdominal pain, constipation and bleeding per rectum had a non-significant increased risk for colonic diverticulosis. The presence of diverticulosis was not significantly correlated with age in symptomatic patients ($P = 0.62$) or asymptomatic persons ($P = 0.52$) (Figure 2).

DISCUSSION

In this study, colonic diverticulosis was identified in nearly 30% of DCBE examinations performed in Thai adults. Right-sided diverticulosis was found more frequently than left-sided diverticulosis. Our findings of colonic diverticulosis are consistent with other observations; in which right-sided colonic diverticulosis is most commonly involved in Asians, whereas sigmoid diverticulosis predominates in Western populations[6,7,15]. Compared with a previous hospital-based study of colonic diverticulosis in Bangkok in the 1980s[11], the present study revealed a markedly higher rate of this condition, but a similar proportion of disease in relatively young individuals. It is difficult to explain why there is a relatively high frequency of colonic diverticulosis in young Thai adults. It is possible that, apart from some differences in dietary intake and lifestyle, racial and genetic predisposition could play an important role in the development of colonic diverticulosis[16]. Apparently, genetic influences on the development of diverticulosis in Asian populations have a stronger impact than those in Western populations, especially for right-sided colonic diverticulosis[17].

Moreover, we found no significant difference in the rate of colonic diverticulosis detected by DCBE between genders, which is consistent with several recent reviews of the literature[8,17,18]. However, there have been a few reports of an increased risk of colonic diverticulosis in males[19,20]. In addition, we did not identify a significant correlation between the presence of diverticulosis and age. Notably, the frequency of pancolonic diverticulosis in our study was 3.4%, which was fairly constant among the different age groups. In contrast to these findings, many authors have repeatedly reported that the prevalence of diverticulosis increases with age[4,21,22]. An interesting study by Takano *et al*[13] also showed that diverticulosis progressed with time from the proximal colon to the distal colon. Although the prevalence and extent of colonic diverticulosis is largely age-dependent, its widespread appearance in the Asian population could be as early as adolescence[20] with peak prevalence at the age of 50-60 years[10]. This could, in part, explain our findings of a relatively high rate of colonic diverticulosis in fairly young age groups; therefore, we did not identify a significant increment in colonic diverticulosis in advanced age groups.

With regard to cecal diverticulosis which involves multiple lesions, we found 42 cases of solitary cecal diverticulum; accounting for 1.5% of all DCBEs studied. Solitary cecal diverticulum is a fairly rare and asymptomatic lesion unless it becomes hemorrhagic or inflamed (mimicking acute appendicitis). Its incidence in Asian populations seems higher than that in Western popula-

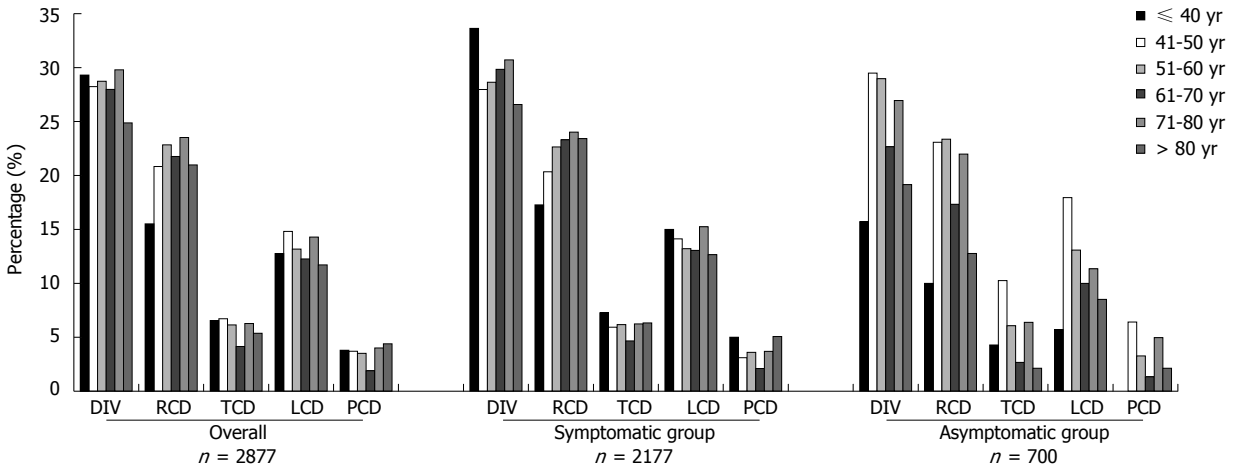

Figure 2 Pattern and distribution of colonic diverticulosis between asymptomatic individuals and symptomatic individuals stratified by age group. DIV: Diverticulosis; RCD: Right-sided colonic diverticulosis; TCD: Transverse colonic diverticulosis; LCD: Left-sided colonic diverticulosis; PCD: Pancolonic diverticulosis.

tions[10,23]. We also identified 12 cases (0.4%) of rectal diverticulum which was exclusively associated with the presence of sigmoid diverticulosis. The true incidence and pathogenesis of rectal diverticulum remain unknown as it is rarely reported[24]. As such lesions were present together with sigmoid colon diverticula, rectal and sigmoid diverticulosis may share the same pathogenesis.

More interestingly, we found a single 5-cm diverticulum in the sigmoid colon in a 51-year-old healthy male. The giant diverticulum was first described in 1946, and to date, fewer than 200 cases have been reported in the literature[25,26]. It is mainly found in the sigmoid colon, and can be divided into 3 distinct histological types: true diverticulum, false diverticulum, and pseudo-diverticulum (scar tissue without any colonic wall layer)[27]. Management of a giant diverticulum depends on the patient's symptoms and underlying disease. Diverticulectomy or segment resection of the affected colon is the favored choice of treatment in symptomatic patients.

Lastly, we demonstrated that the DCBE examinations performed in patients with some gastrointestinal symptoms (*e.g.*, bowel habit change, constipation, abdominal pain and hematochezia) revealed a higher prevalence of colonic diverticulosis than those performed in asymptomatic individuals. It is obvious that many patients with colonic diverticulosis experience chronic gastrointestinal symptoms at some time in their life[28]. However, it is difficult to know whether colonic diverticulosis is a cause or a result of such symptoms.

In conclusion, the present study examined the frequency and distribution of colonic diverticulosis from a relatively large number of fluoroscopic DCBEs performed in Thai adults. Colonic diverticulosis was identified in nearly 30% of DCBE examinations. Right-sided diverticulosis was more common than left-sided diverticulosis. There was no association between the presence of diverticulosis and gender or age. Colonic diverticulosis was more commonly reported in patients with some gastrointestinal symptoms, especially those with change in bowel habit.

COMMENTS

Background

Colonic diverticulosis is a common gastrointestinal condition. The prevalence and distribution of colonic diverticulosis differ among ethnic groups and lifestyles; left-sided diverticulosis is more common in Western and developed countries, while right-sided diverticulosis is more prevalent in Asian and developing countries. Moreover, the characteristics of colonic diverticulosis have changed over time.

Research frontiers

Although some data on colonic diverticulosis from Asian countries are available, the information on colonic diverticulosis in Southeast Asia is limited and some are outdated. Double contrast barium enema (DCBE) is a reliable investigation tool for demonstrating the presence and extent of colonic diverticulosis.

Innovations and breakthroughs

This paper demonstrates that colonic diverticulosis was identified in 28.5% of DCBEs performed in Thai adults. Right-sided diverticulosis was more common than left-sided diverticulosis. Pancolonic diverticulosis was found in 3.4% of patients. There was no association between the presence of diverticulosis and gender or age, however, DCBE examinations performed in patients with some gastrointestinal symptoms revealed the frequent occurrence of colonic diverticulosis compared with those performed in asymptomatic individuals. Change in bowel habit was strongly associated with the presence of diverticulosis.

Applications

The study results show that the occurrence of colonic diverticulosis in Thailand, a developing country in Asia, is surprisingly prominent and markedly higher than that previously reported from a hospital survey in Bangkok approximately 30 years ago. In addition, the rate of colonic diverticulosis in the present study was equal in the different age groups *i.e.*, its widespread appearance could be seen as early as adolescence. These findings may urge physicians to include or consider colonic diverticular disease as one of the causes of gastrointestinal symptoms in every patient, including young individuals.

Terminology

Colonic diverticulosis is usually described as the presence of outpouching(s) of the mucosa and submucosa through a weak area of the large intestine. When a diverticulum (or multiple diverticula) becomes symptomatic, infected or bleeding, this gastrointestinal condition may be called "colonic diverticular disease".

Peer review

This is a good descriptive study in which authors analyze the pattern and distribution of colonic diverticulosis from a third world country, where the frequency of such a condition is expected to be low. In fact, this study showed an unexpectedly high number of colonic diverticulosis in Thai adults. The distribution of colonic diverticulosis is brilliantly shown in great details. The results are also interesting and suggest that colonic diverticulosis can be seen in adolescence as well as it occurrence is not age-dependent. Some findings are different from those shown in Western populations.

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BRIEF ARTICLE

Intestinal stem cell marker LGR5 expression during gastric carcinogenesis

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Abstract

AIM: To investigate the differential expression of leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) in gastric cancer tissues and its significance related to tumor growth and spread.

METHODS: Formalin-fixed biopsy specimens of intestinal metaplasia (n = 90), dysplasia (n = 53), gastric adenocarcinoma (n = 180), metastases in lymph nodes and the liver (n = 15), and lesion-adjacent normal gastric mucosa (controls; n = 145) were obtained for analysis from the Peking University Cancer Hospital's Department of Pathology and Gastrointestinal Surgery tissue archives (January 2003 to December 2011). The biopsied patients' demographic and clinicopathologic data were retrieved from the hospital's medical records database. Each specimen was subjected to histopathological typing to classify the tumor node metastasis (TNM) stage and to immunohistochemistry staining to detect the expression of the cancer stem cell marker LGR5. The intergroup differences in LGR5 expression were assessed by Spearman's rank correlation analysis, and the relationship between LGR5 expression level and the patients' clinicopathological characteristics was evaluated by the χ^2 test or Fisher's exact test.

RESULTS: Significantly more gastric cancer tissues showed LGR5$^+$ staining than normal control tissues (all P < 0.01), with immunoreactivity detected in 72.2% (65/90) and 50.9% (27/53) of intestinal metaplasia and dysplasia specimens, respectively, 52.8% (95/180) of gastric adenocarcinoma specimens, and 73.3%% (11/15) of metastasis specimens, but 26.9% (39/145) of lesion-adjacent normal gastric mucosa specimens. Comparison of the intensity of LGR5$^+$ staining showed an increasing trend that generally followed increasing dedifferentiation and tumor spread (normal tissue < dysplasia, < gastric adenocarcinoma < metastasis; all P < 0.001), with the exception of expression level detected in intestinal metaplasia which was higher than that in normal gastric tissues (P < 0.001). Moreover, gastric cancer-associated enhanced expression of LGR5 was

found to be significantly associated with age, tumor differentiation, Lauren type and TNM stage (Ⅰ + Ⅱ *vs* Ⅲ + Ⅳ) (all $P < 0.05$), but not with sex, tumor site, location, size, histology, lymphovascular invasion, depth of invasion, lymph node metastasis or distant metastasis. Patients with LGR5$^+$ gastric cancer specimens and without signs of metastasis from the original biopsy experienced more frequent rates of recurrence or metastasis during follow-up than patients with LGR5$^-$ specimens ($P < 0.05$).

CONCLUSION: Enhanced LGR5 is related to progressive dedifferentiation and metastasis of gastric cancer, indicating the potential of this receptor as an early diagnostic and prognostic biomarker.

Key words: Leucine-rich repeat-containing G protein-coupled receptor 5; Cancer stem cell; Gastric cancer; Intestinal metaplasia; Tumorigenesis

Core tip: This is the first study to evaluate the expression of leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5), a putative cancer stem cell marker, in progressive stages of gastric carcinogenesis. The observation of increasing LGR5 expression in human gastric cancer lesions, following the loss of differentiation (from dysplastic to gastric cancer cases) and risk of spread (metastatic cases), suggests that this receptor may represent an important biomarker for early detection of patients at higher risk for gastric tumorigenesis. The observed distinctive expression pattern of LGR5 in intestinal metaplasia suggests that these cells may represent a precancerous condition but not carcinoma precursors.

Zheng ZX, Sun Y, Bu ZD, Zhang LH, Li ZY, Wu AW, Wu XJ, Wang XH, Cheng XJ, Xing XF, Du H, Ji JF. Intestinal stem cell marker LGR5 expression during gastric carcinogenesis. *World J Gastroenterol* 2013; 19(46): 8714-8721 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i46/8714.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i46.8714

INTRODUCTION

Gastric cancer (GC) is one of the most common cancers worldwide, yet the majority of GC-related deaths occur in less developed countries, including China and other Asian nations[1,2]. Studies to elucidate the tumorigenic processes underlying GC development have revealed a multistep sequential process involving normal gastric tissue progression to chronic gastritis, atrophy, intestinal metaplasia, dysplasia, and carcinoma, with or without metastatic potential[3]. This model supports the possibility of a stepwise accumulation of genetic alterations affecting expression of key molecules, possibly having direct or indirect (*i.e.*, signaling pathways) functional effects on cell growth and movement.

The stem cell origin hypothesis of carcinomas has gained much research attention in the recent decade. Cancer stem cells (CSCs), which express a distinctive profile of cell type-specific surface markers[4], have been detected in a broad range of clinical cancer specimens, including hematological malignancies and solid tumors of the breast, lung, ovary, liver, prostate, pancreas, skin, brain and colon[5-13]. However, few studies to date have investigated the presence of CSCs in GC lesions, and their role in GC tumorigenesis remains largely unknown.

The leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5, also known as GPR49) has been proposed as a marker of GC-related stem cells. Under normal conditions, LGR5 is expressed primarily on intestinal stem cells, where it functions as a transducer of Wnt signaling[14,15]. Murine-based investigations to uncover the role of LGR5 in cancer development and progression have also demonstrated its expression on rare, scattered cells in the eye, brain, stomach, mammary gland and reproductive organs[16] and showed that LGR5$^+$ stem cells were much more effective in producing tumorigenesis than more differentiated (LGR5$^-$) cells[17]. In humans, LGR5$^+$ cells have been detected in both the population of crypt stem cells (precursor cells) and gastric mucosal lesions that progressed to cancer[18].

A functional study of LGR5-expressing cells and their age-related distribution using a mouse model revealed that its expression was localized to the base of prospective corpus and pyloric glands in neonatal stomach but predominantly restricted to the base of mature pyloric glands in adult stomach, and demonstrated that a single LGR5$^+$ cell could efficiently generate long-lived organoids resembling mature pyloric epithelium *in vitro*[19]. While the collective findings have increased interest in developing LGR5 as a universal epithelial CSC marker for clinical use[18], the loss of restriction to the stem cell niche is considered an early event in the premalignant transformation of stem cells and suggests that this protein may also be a key contributor to carcinogenesis.

Although many previous studies have investigated the association of perturbed LGR5 expression with tumorigenesis, very few have reported on the differential expression of LGR5 and its role in the multistep sequential process of GC development. Therefore, the present study analyzed LGR5 expression in human clinical specimens of gastric tissues from the non-cancerous condition through gastric adenocarcinoma and in GC-related lymph nodes and liver metastases, and evaluated the relationship between differential LGR5 expression and clinicopathological features. The findings from this study will provide novel insights into the carcinogenic process of GC from the perspective of the stem cell origin hypothesis.

MATERIALS AND METHODS

Patients and tissue samples

Formalin-fixed/paraffin-embedded specimens of in-

testinal metaplasia (n = 90), dysplasia (n = 53), gastric adenocarcinoma (n = 180), metastases in lymph nodes and the liver (n = 15), and lesion-adjacent normal gastric mucosa (controls; n = 145) were obtained for analysis from the Peking University Cancer Hospital's Department of Pathology and Gastrointestinal Surgery tissue archives (January 2003 to December 2011). All specimens had been obtained during endoscopic biopsy or surgical resection. Each specimen was analyzed by routine histopathological analysis and was classified according to the pathological criteria published by the World Health Organization (4th edition) and the tumor node metastasis (TNM) staging system of the American Joint Committee on Cancer Staging Manual (7th edition) and the Japanese Gastric Cancer Association Guidelines (3rd edition).

The biopsied patients' demographic and clinicopathologic characteristics (during the clinical management and follow-up periods) were retrieved from the hospital's electronic records database. If a patient had no record of death but lacked follow-up data, the patient's general practitioner was contacted to obtain the information. None of the GC patients had synchronous cancers or previous gastrointestinal diseases, nor had undergone abdominal surgery, chemotherapy or radiotherapy prior to specimen collection.

This study was performed with pre-approval from the Ethics Committee of Peking University Cancer Hospital. Informed consent allowing for investigative use of tissue samples had been provided by each patient.

Immunohistochemical analysis

Specimen sections (4 μm thickness) were mounted on poly-*L*-lysine coated slides, deparaffinized in xylene, and rehydrated in a descending ethanol-to-water gradient series. Endogenous peroxidase was blocked by exposure to 3% H_2O_2 for 10 min, followed by antigen retrieval via pressurized heating in EDTA buffer (Zhongshan Biotechnology Inc., Beijing, China) for 5 min. After cooling to room temperature, non-specific sites were blocked by exposure to 10% goat blood serum. LGR5 immunodetection was carried out by incubating with purified rabbit polyclonal antibody (AP2745d; Abgent, San Diego, CA, United States), followed by two-step diaminobenzidine visualization (GK500705; Dako, Glostrup, Denmark).

The immunostained sections were counterstained with hematoxylin for 40 s, rinsed in water, dehydrated in an ascending water-to-ethanol gradient series followed by clearance with xylene, and permanently cover-slipped. Negative controls were created using the same procedure but without addition of primary antibody.

Evaluation of immunostaining

The processed immunostained sections were examined by light microscopy. Two experienced pathologists (Sun Y and Dong B), working independently and blinded to the corresponding clinical data, evaluated each sample to calculate and score the percentage of $LGR5^+$ cells [none (negative, -): 0%, 1%-25%: 1, 25%-50%: 2, and > 50%: 3] and to score the intensity of cytoplasmic staining (no staining: 0, mild: 1, moderate: 2, and strong: 3; with the highest intensity score being assigned when > 10% of cells stained with that intensity). Adding the percentage and intensity scores provided a composite expression score (0-6), which was defined as: weakly positive (+): 1-2, moderately positive (++): 3-4, and strongly positive (+++): 5-6. For statistical analysis, a composite score of 0 was classified as negative and 1-6 as positive, with $\leqslant 2$ ranked as low expression and $\geqslant 3$ ranked as high expression.

Statistical analysis

All statistical analyses were carried out using the SPSS software statistical package (version 20.0; SPSS Inc., Chicago, IL, United States). The differences in LGR5 expression between the gastric tissue types were analyzed by Spearman's rank correlation analysis. The relationships between LGR5 differential expression and clinicopathological characteristics were evaluated by the χ^2 test or Fisher's exact test. A two-sided P-value < 0.05 was considered statistically significant.

RESULTS

LGR5 expression and distribution in normal gastric mucosa

Immunostaining of LGR5 showed a predominant localization to the cytoplasm or on the cell membrane in normal gastric mucosa specimens. Morphologically, the $LGR5^+$ cells were localized to the mucous neck region at the base of the gastric crypts between the foveolae and glands (Figure 1A and B). The positive-staining percentages are presented in Table 1.

Differential LGR5 expression in GC-related tissues during tumorigenesis

Immunodetection of LGR5 in GC-related tissues, progressing from non-neoplastic epithelia to gastric cancer and finally metastasis, showed an increasing trend in the number and intensity of $LGR5^+$ cells (all *vs* normal gastric mucosa specimens and *vs* the different GC-related tissues, P < 0.05; Table 1). In addition, the significantly enhanced LGR5 expression in dysplasia specimens (P = 0.019) was largely accounted for by the specimens with low grade dysplasia (roughly twice that of the high grade dysplasia specimens). The GC-related enhanced LGR5 expression was also greater in specimens from patients with lower clinical stage (TNM stages Ⅰ + Ⅱ > Ⅲ + Ⅳ) and the majority of GC cases showed weak staining (with strong cytoplasmic or membranous immunodetection < moderate staining < weak staining < no staining; Figure 1C-J). Morphologically, the distribution of $LGR5^+$ cells was uneven and inhomogeneous in the GC-related specimens and occurred in cohesive patches of a variable number of tumor cells.

Association of immunodetected LGR5 expression with clinicopathological features of GC patients

The patients' demographic and clinicopathologic features

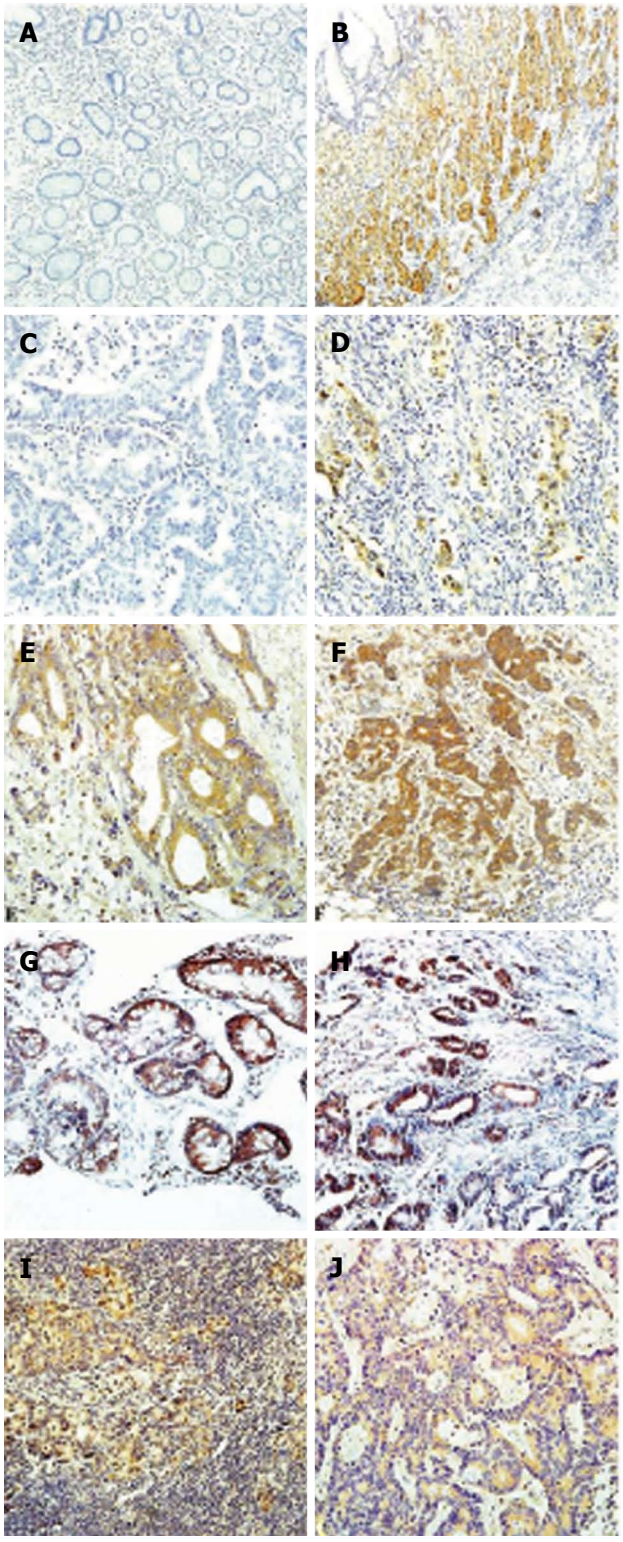

Figure 1 Immunodetected differential LGR5 expression in gastric tissues, following progression of tumorigenesis, and in distant metastases. Representative samples are shown from leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5)$^-$ (A) and LGR5$^+$ (B) normal gastric normal tissues, LGR5$^-$ (C) and LGR5$^+$ (D-F) gastric cancer (GC) tissues with weak, moderate and strong expression, LGR5$^+$ gastric intestinal metaplasia and dysplasia tissues (G, H), and LGR5$^+$ lymph node and liver metastases (I, J). A, C-J: Magnification: × 200; B: Magnification: × 100.

Table 1 LGR5 immunostaining in gastric cancer-related gastric tissues and metastases *n* (%)

Pathological type	Total, *n*	LGR5 expression		*P* value
		Negative	Positive	
Normal tissue	145	106 (73.1)	39 (26.9)	0.000
Dysplasia grade	53	26 (49.1)	27 (50.9)	
Low	25	8 (32.0)	17 (68.0)	0.019
High	28	18 (64.3)	10 (35.7)	
TNM stage	180	85 (47.2)	95 (52.8)	
Ⅰ-Ⅱ	71	27 (38.0)	44 (62.0)	
Ⅲ-Ⅳ	109	58 (53.2)	51 (46.8)	
Metastases	15	4 (26.7)	11 (73.3)	
Lymph node	5	1 (20.0)	4 (80.0)	
Liver	10	3 (30.0)	7 (70.0)	

LGR5: Leucine-rich repeat-containing G protein-coupled receptor 5; TNM: Tumor, nodes, metastasis.

Table 2 Association of immunodetected LGR5 expression with clinicopathological features of gastric cancer patients *n* (%)

Clinicopathological feature	LGR5 expression		*P* value
	Negative	Positive	
Sex			0.072
Male	56 (43.1)	74 (56.9)	
Female	29 (58.0)	21 (42.0)	
Age, yr			0.005
≤ 60	48 (58.5)	34 (41.5)	
> 60	37 (37.8)	61 (62.2)	
Location in stomach			0.657
Upper	15 (40.5)	22 (59.5)	
Mid	17 (47.2)	19 (52.8)	
Lower	45 (49.5)	46 (50.5)	
Lesion size in cm			0.612
≤ 4	33 (42.9)	44 (57.1)	
> 4	32 (47.1)	36 (52.9)	
Differentiation			0.006
Differentiated	31 (36.5)	54 (63.5)	
Undifferentiated	54 (56.8)	41 (43.2)	
Histological type			0.579
Adenocarcinoma	67 (46.2)	78 (53.8)	
Others	18 (51.4)	17 (48.6)	
Lauren type			0.035
Intestinal	48 (41.4)	68 (58.6)	
Diffuse/other	37 (57.8)	28 (42.2)	
Lymphovascular invasion			0.288
No	43 (43.9)	55 (56.1)	
Yes	42 (51.9)	39 (48.1)	
Depth			0.833
T1-T2	19 (48.7)	20 (51.3)	
T3-T4	66 (46.8)	75 (53.2)	
Lymph node metastasis			0.934
No	19 (47.5)	21 (52.5)	
Yes	65 (46.8)	74 (53.2)	
Metastasis			0.160
No	71 (45.2)	86 (54.8)	
Yes	14 (60.9)	9 (39.1)	
TNM			0.046
Ⅰ-Ⅱ	27 (38.0)	44 (62.0)	
Ⅲ-Ⅳ	58 (53.2)	51 (46.8)	

LGR5: Leucine-rich repeat-containing G protein-coupled receptor 5; TNM: Tumor, nodes, metastasis.

are summarized in Table 2. There were more males than females (130 *vs* 50), but the percentage of LGR5$^+$ immunodetection was similar between the two and sex was not found to be significantly correlated with LGR5 expression in the GC-related specimens. The overall patients

Table 3 LGR5 expression in gastric cancer tissues of various differentiation *n* (%)

Tissue	LGR5 expression		*P* value
	Negative	Positive	
Intestinal metaplasia	25 (27.8)	65 (72.2)	0.000
Normal tissue	106 (73.1)	39 (26.9)	
Dysplasia with IM			0.004
Yes	3 (18.8)	13 (81.2)	
No	23 (62.2)	14 (37.8)	
Lauren type			0.035
Intestinal	48 (41.4)	68 (58.6)	
Diffuse/other	37 (57.8)	28 (42.2)	
Intestinal type GC			0.019
Metastasis or recurrence	6 (12.5)	21 (31.3)	
No metastasis or recurrence	42 (87.5)	46 (68.7)	

LGR5: Leucine-rich repeat-containing G protein-coupled receptor 5; GC: Gastric cancer; IM: Intestinal metaplasia.

ranged in age from 22-87 years old (median: 62 years old), and age was found to be significantly associated with LGR5$^+$ immunodetection in GC-related specimens (P = 0.005). In addition, differentiation (P = 0.006), Lauren type [P = 0.035, with intestinal type having significantly more LGR5$^+$ cells than the diffuse/other types (58.6% *vs* 42.2%)] and TNM stage (Ⅰ + Ⅱ *vs* Ⅲ + Ⅳ, P = 0.046) were also correlated significantly with LGR5$^+$ immunodetection, but tumor site, location, size, histology, lymphovascular invasion and depth of invasion were not.

Analysis of the follow-up data showed that GC patients without metastases at surgery but with LGR5$^+$ staining specimens experienced a higher rate of recurrence or metastasis than their counterparts with LGR5$^-$ staining specimens (87.35% *vs* 12.7%, P = 0.020). However, the presence of metastases at surgery was not correlated with LGR5$^+$ immunodetection (both P > 0.05; Table 2). The specimens from patients with intestinal type GC also showed a significantly higher rate of LGR5$^+$ immunodetection than those from patients with diffuse or mixed types GC (P = 0.035), and LGR5$^+$ immunodetection in intestinal type GC was associated with more frequent rates of recurrence or metastasis after surgery (P = 0.019; Table 3).

Association of LGR5 expression with transformation of intestinal metaplasia tissues

As shown in Table 3, intestinal metaplasia specimens showed a significantly higher rate of LGR5$^+$ immunodetection than normal gastric tissues (P = 0.000). Moreover, dysplasia specimens with intestinal metaplasia had a significantly higher rate of LGR5$^+$ immunodetection than those without (P = 0.004).

DISCUSSION

Using a standard immunohistochemistry-based method, the differential expression pattern of the putative CSC marker LGR5 in progressively tumorigenic clinical specimens of GC was demonstrated. In particular, an increasing trend was observed in LGR5$^+$ staining intensity that generally followed increasing dedifferentiation and tumor spread (normal tissue < dysplasia < gastric adenocarcinoma < metastasis).

The adenoma-carcinoma progression sequence is well known in colorectal cancer and esophageal adenocarcinoma, and is becoming more generally accepted as the likely mode of tumorigenesis in the gastrointestinal tract as well[20-23]. Recent findings from studies in mammalian (mouse) model systems and with human GC specimens have demonstrated that GC progenitor cells are derived from multipotent stem cells in the highly regenerative and proliferative regions of the stomach, including the isthmus and fundic gland-rich neck[24,25]. Indeed, the subpopulation of stem cells with high LGR5 expression were shown to have the capability to reconstitute crypt structures *in vitro*[26], and LGR5 has been detected on progenitor cells in human gastric mucosa crypts[27,28].

As stated in the Introduction, the multitude of signaling factors that comprise this multistep progression model of GC tumorigenesis also represent a plethora of targets for improved detection and treatment methods. The occurrence of gastric epithelial dysplasia is a well-characterized precursor event to GC, and is currently considered the most dependable marker for such cancer risk. A prospective longitudinal study of gastric epithelial dysplasia and development of GC indicated that high grade dysplasia is associated with rapid development of intestinal type GC[29]. This finding is in line with the current study's observation of similar LGR5$^+$ immunodetection rates in dysplasia and gastric carcinoma specimens (with a slightly higher rate in the latter), and higher rates in well to moderately differentiated intestinal type and lower-staged gastric cancers.

The dynamic undulation of immunodetected LGR5 expression observed in the low clinical stage (enhanced in Ⅰ-Ⅱ) to the high clinical stage (reduced in Ⅲ-Ⅳ) to metastasis (again enhanced) agrees with a previously reported profile of LGR5 expression in tumorigenesis of endometrial, colorectal and ovarian carcinomas (with the high expression demonstrated during the initial stages, being down-regulated in fully developed tumors)[30,31]. Collectively, these findings support the hypothesized clonal selection model of putative stem cells leading to carcinogenesis[32]. In particular, the results from the current study suggest that overexpression of LGR5 may be an early event in tumorigenesis and that immunodetection of such protein is achieved with good reproducibility and tracks with differentiation of tumor specimens.

From a mechanistic perspective, the tumorigenic-related expression profile observed in the current study suggests the existence of a potential tumor promoter regulating LGR5. However, it is important to consider the unexpected observation of higher immunodetected LGR5 expression in low grade dysplasia than in high grade dysplasia; similar results were also reported from another study of esophageal dysplasia lesions[20]. A possible explanation of this result is the fact that the cur-

rent morphologic criteria for different grade dysplasias include a mix of architectural and cytologic features and do not consider functional characteristics[33]. Indeed, low grade dysplasia preserves some of the functions of intestinal metaplasia, which underlies the risk of misdiagnosis for these two conditions[34]. Previous studies have addressed this confusing issue, proposing that the increased amounts of high-intensity LGR5$^+$ cells that are observed in dysplasia may represent a stem cell population that is prone to becoming CSCs[35,36].

Other intriguing findings from the current study are the higher amounts of LGR5$^+$ cells detected towards the crypt base or in the invasive tumor front during the development and progression of GC (although the change in differential expression did not reach statistical significance) and in metastases (both local and distant). Brablez *et al*[37] hypothesized that tumor progression is mediated by two types of CSCs with distinct functions. The first was proposed as a stationary cancer stem (SCS) cell population, which would be present in the area for cell differentiation but which would not promote metastasis. The second was proposed as a migrating (or mobile) cancer stem (MCS) cell population, which may be derived from the SCS cells and located primarily at the invasive tumor front, and which would drive metastasis. Therefore, the observed shift in distribution of LGR5$^+$ cells towards the invasive tumor front that accompanied the development and metastasis of GC in the current study may be related to such MCS cells. This notion may also be in line with the current study's observation of GC patients with LGR5$^+$ intestinal type specimens being at higher risk of recurrence or metastasis after surgery.

Previous studies have demonstrated that Wnt signaling regulates stemness and organ development, as well as the process of epithelial to mesenchymal transition (EMT) that increases the metastatic potential of disseminated cancer cells[38,39]. In addition, EMT may also restart the growth and differentiation programs of stem cells at metastatic sites[37,40]. Studies of human colorectal cancer have demonstrated that aberrant Wnt signaling not only triggers early steps of intestinal carcinogenesis but also malignant tumor progression towards invasive carcinomas and metastasis[41-43]. Therefore, LGR5 (as a Wnt target and a stem cell marker) plays an important role in initiating tumor growth and driving distant metastasis. These functions of LGR5 may also explain the findings in the current study of LGR5$^+$ GC patients without evidence of metastases during the initial surgical treatment being at a greater risk of recurrence or metastasis.

Interestingly, the LGR5-immunodetected expression had higher intensity in gastric intestinal metaplasia, dysplasia with intestinal metaplasia, and intestinal type GC than in the normal tissues examined in the current study; all of these GC-related lesions have the potential to manifest intestinal type differentiation. Intestinal metaplasia has been shown to originate from stem cells of the isthmus, and the crypts possess multiple stem cells[44,45]. Although intestinal metaplasia is regularly detected in the antrum of patients with gastritis and duodenal ulcers related to *Helicobacter pylori* infection, these patients very rarely develop gastric carcinoma[46]. Similarly, Tatematsu *et al*[47] suggested that gastric/intestinal mixed type intestinal metaplasia might be the consequence of abnormal differentiation of stem cells that are capable of producing both gastric and intestinal types of cells.

Only the relatively rare type Ⅲ intestinal metaplasia has been identified as a risk marker for the development of gastric carcinoma, being classified as "low grade dysplasia"[34]. The related findings in our study suggest that intestinal metaplasia may be a precancerous condition, but not a precursor for gastric carcinoma (possibly with the exception of some rare types). Thus, LGR5 may represent a unique and sensitive marker of intestinal stem cells and may be closely related to the intestinal type of GC.

In conclusion, the immunodetectable expression pattern of LGR5, a CSC-related gene, increasing from normal tissues to lesions of dysplasia, gastric carcinoma and finally metastases, suggests potential for this protein to serve as an important biomarker for early detection of patients at higher risk for gastric tumorigenesis. Furthermore, as an intestinal stem cell marker, differential LGR5 expression in conjunction with development of intestinal metaplasia may represent a precancerous condition, but not a carcinoma precursor.

COMMENTS

Background

Cancer stem cells (CSCs) may be the source of various carcinomas, including gastric cancer (GC), and are identifiable by clinically detectable profiles of cell type-specific surface markers. The leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5), a target of Wnt signaling, is primarily expressed on normal intestinal stem cells and has been suggested as a putative CSC marker (and contributor to GC tumorigenesis) according to its differential expression on crypt stem cells (precursor cells) and gastric lesions that progress to cancer. Accumulated evidence has suggested roles for LGR5 in both cancer development and progression. Recent studies have also indicated that LGR5 may be a potential marker of gastrointestinal stem cells in humans and that loss of restriction to the stem cell niche is likely an early event in the premalignant transformation of stem cells.

Research frontiers

The differential protein expression of LGR5 in normal gastric tissue, intestinal metaplasia and dysplasia specimens, gastric carcinomas, and distant metastases was determined by immunohistochemistry to provide insights into its potential as a clinical marker for early GC detection. Furthermore, the differential LGR5 expression observed in conjunction with development of intestinal metaplasia suggests that this phenomenon represents a precancerous condition, but not a carcinoma precursor.

Innovations and breakthroughs

An increasing trend in intensity of LGR5 expression was detected in GC-related tissues, following the well-recognized sequential development from normal tissue to dysplasia to gastric carcinoma and finally metastasis, with the exception of the intestinal metaplasia state. The differential expression of LGR5 detected in GC by immunohistochemistry appeared to be significantly associated with age, differentiation, Lauren type, and tumor node metastasis stage. The LGR5$^+$ cells detected in intestinal metaplasia specimens were more prevalent than those detected in normal gastric tissues, and the data indicated that intestinal metaplasia may manifest from differentiation of a population of abnormal stem cells with high expression of LGR5, but may not represent a carcinoma precursor. Collectively, these data indicate that LGR5 expression may serve as an important biomarker for early detection of patients at higher risk for gastric tumorigenesis, and may be a candidate target for future individualized therapeutic

strategies.

Applications

The current poor prognosis of GC is largely associated with the low rate of early diagnosis. The findings from this study of human clinical samples of GC lend to a recommendation that LGR5 should be the focus of further studies to develop its potential as a biomarker for early detection of patients at higher risk for GC and as a manipulable intestinal stem cell marker target for improved management of GC cases.

Terminology

The leucine-rich repeat-containing G protein-coupled receptor 5 is expressed primarily on intestinal stem cells, where it functions as a transducer of Wnt signaling. Cancer stem cells, which express a distinctive profile of cell type-specific surface markers, have been detected in a broad range of clinical cancer specimens and are the basis of the stem cell origin hypothesis of cancer. Gastric cancer development is a multistep sequential process involving normal gastric tissue progression to chronic gastritis, atrophy, intestinal metaplasia, dysplasia, and carcinoma, with or without metastatic potential.

Peer review

This study determined the GC-related expression profile of the putative CSC marker LGR5, using standard immunohistochemistry to detect expression in human clinical samples of normal gastric tissue, intestinal metaplasia, dysplasia, gastric carcinoma, and distant metastases. The observed increasing trend in differential LGR5 expression following progressive tumorigenesis to metastasis suggests that this protein may serve as an important biomarker for early detection of patients at higher risk for gastric tumorigenesis. The data also implicate a role for LGR5 as an intestinal stem cell marker and suggest that intestinal metaplasia may be a precancerous condition but not a carcinoma precursor. The study is well controlled and provides novel insights into this life-threatening disease.

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BRIEF ARTICLE

Conservative treatment of early postoperative small bowel obstruction with obliterative peritonitis

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Abstract

AIM: To investigate the effect of somatostatin and dexamethasone on early postoperative small bowel obstruction with obliterative peritonitis (EPSBO-OP).

METHODS: This prospective randomized study included 70 patients diagnosed with EPSBO-OP from June 2002 to January 2009. Patients were randomized into two groups: a control group received total parenteral nutrition and nasogastric (NG) tube feeding; and an intervention group received, in addition, somatostatin and dexamethasone treatment. The primary endpoints were time to resolution of bowel obstruction and length of hospital stay, and the secondary endpoints were daily NG output and NG feeding duration, treatment-related complications, postoperative obstruction relapse, and patient satisfaction.

RESULTS: Thirty-six patients were allocated to the intervention group and 34 to the control group. No patient needed to undergo surgery. Patients in the intervention group had an earlier resolution of bowel obstruction (22.4 ± 9.1 *vs* 29.9 ± 10.1 d, $P = 0.002$). Lower daily NG output (583 ± 208 *vs* 922 ± 399 mL/d, $P < 0.001$), shorter duration of NG tube use (16.7 ± 8.8 *vs* 27.7 ± 9.9 d, $P < 0.001$), and shorter length of hospital stay (25.8 *vs* 34.9 d, $P = 0.001$) were observed in the intervention group. The rate of treatment-related complications ($P = 0.770$) and relapse of obstruction ($P = 0.357$) were comparable between the two groups. There were no significant differences in postoperative satisfaction at 1, 2 and 3 years between the two groups.

CONCLUSION: Somatostatin and dexamethasone for EPSBO-OP promote resolution of obstruction and shorten hospital stay, and are safe for symptom control without increasing obstruction relapse.

Key words: Dexamethasone; Intestinal obstruction; Parenteral nutrition; Postoperative period; Somatostatin

Core tip: This prospective study revealed that somatostatin and dexamethasone, when used in combination, promoted the resolution of small bowel obstruction and shortened length of hospital stay in patients with early postoperative small bowel obstruction due to obliterative peritonitis. Somatostatin and dexamethasone were effective in symptom control in this population.

Gong JF, Zhu WM, Yu WK, Li N, Li JS. Conservative treatment of early postoperative small bowel obstruction with obliterative peritonitis. *World J Gastroenterol* 2013; 19(46): 8722-8730 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i46/8722.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i46.8722

INTRODUCTION

Early postoperative small bowel obstruction (EPSBO) with obliterative peritonitis (EPSBO-OP), or "frozen abdomen", (also known as early postoperative inflammatory small bowel obstruction[1,2], is caused by dense, vascular and inseparable inflammatory adhesions in response to multiple sequential laparotomies, surgery for enterocutaneous fistula (ECF), or extensive adhesiolysis[3-6]. Patients with EPSBO-OP may often have a combination of partial mechanical obstruction and diffuse small bowel and colonic ileus. Surgery attempting to lyse the adhesions in these patients is unsuitable due to the high risk of iatrogenic injuries such as ECF or massive small bowel resection[7].

The traditional approach to managing these patients is total parenteral nutrition (TPN) and observation, and most obstructions are relieved spontaneously[8]. However, it often takes a long period (*i.e.*, several weeks to months) before the bowel function recovers[9], and it is associated with high costs and high risk of PN-related complications. Patients have to tolerate prolonged nasogastric (NG) suction and fluid loss, which can also create discomfort and complications.

Somatostatin is well known for its antisecretory function in the intestinal epithelium, and clinical studies have suggested that it may be useful for symptomatic relief and treatment of bowel obstruction[10,11]. Dexamethasone is a frequently used synthetic corticosteroid that reduces intraperitoneal adhesion and inflammatory edema[12,13], and is effective in promoting the resolution of malignant bowel obstruction or obstruction with encapsulating peritoneal sclerosis[14,15]. Based on their mechanisms of action and results of previous studies, we hypothesized that these two drugs, when used in combination, would be beneficial in reducing gastrointestinal secretion and promoting the regression of inflammation and adhesion in patients with EPSBO-OP. However, comparative studies of the effect of somatostatin and dexamethasone in EPSBO-OP are lacking.

In the current study, we prospectively analyzed a consecutive series of patients with ESPBO-OP in our department, a tertiary gastrointestinal referral center in China. The aim of the study was to evaluate the effect of somatostatin and dexamethasone on length of hospital stay and symptom control in patients with ESPBO-OP.

MATERIALS AND METHODS

Patients

The diagnostic criteria for ESPBO-OP were: (1) intestinal obstruction that developed 1-4 wk postoperatively after initial recovery of postoperative ileus, as defined previously[16,17]; (2) typical operative history with extensive enterolysis or repeated laparotomy over a short period; (3) absence of severe colicky abdominal pain, but with obstipation, abdominal distension, nausea and vomiting; (4) palpation of a subincisional or whole abdominal mass on physical examination, with only mild or no tenderness on palpation, no peritoneal signs, and low-pitched or no bowel tones; and (5) low or absent air-fluid levels on upright abdominal film, edematous and thickened bowel wall with unclear borders on abdominal computed tomography (CT), and fluid-filled lumen with paucity of gas.

Exclusion criteria included: patients aged < 18 years; patients with terminal disease or presence of metastatic cancer; CT or X-ray film suggesting local adhesions, intussusceptions, volvulus, internal hernia, intra-abdominal abscesses, or hematoma; patients with suspicion of mechanical bowel obstruction, paralytic ileus, or idiopathic pseudo-obstruction; and patients with hypokalemia and retroperitoneal injury that may cause paralytic ileus. On previous laparotomy, all adhesions should have been freed and the possibility of mechanical obstruction, such as anastomotic stenosis or residual malignancy, excluded. All radiographs (X-ray and CT scan) were extrapolated by a specialist in gastrointestinal radiology.

Assignments were based on computer-generated randomizations that were kept in sealed, sequentially numbered envelopes until used. After a diagnosis of EPSBO-OP was made, patients were randomly assigned into one of the two groups: TPN group (T group) or TPN + dexamethasone + somatostatin group (TDS group). The study was approved by the ethics committee of the hospital, and all patients provided written informed consent before enrollment.

Treatment

Nil by mouth and nasogastric tube were introduced for all patients. For patients in the control group (T group), a central venous catheter was placed on admission. After fluid resuscitation and correction of electrolyte abnormalities, patients were infused with TPN from all-in-one bags. The amount of non-protein calories (NPCs) was 20-25 kcal/kg per day or determined by indirect calorimetry. The NPCs consisted of 60%-70% carbohydrate, with the ratio of NPC: nitrogen = 120-140:1. Parenteral antibiotics were administered when leukocytosis was present. The amount of intravenous fluid was adjusted to maintain optimal hydration and sufficient urine output (> 1 L/d).

The duration of NG tube feeding depended on daily output. If daily NG output was < 200 mL for 2 d, the NG tube was clamped. The NG tube was removed if the patient was able to tolerate for 12 h after clamping. After patients resumed oral intake, gastrointestinal prokinetics (mosapride, 5 mg/8 h, Gasmotin; Dainippon Sumitomo Pharma Co. Ltd., Osaka, Japan) was given until discharge.

In the intervention group (TDS group), in addition to the treatment protocol in the control group, somatostatin (Stilamin; Merck-Serono S.A., Geneva, Switzerland) was given at 6 mg/d by continuous intravenous infusion. The criteria for stopping NG tube usage were similar to those for the T group, while somatostatin was stopped within 24 h after the patient defecated or passed gas. Dexameth-

asone sodium phosphate (5 mg/mL, Lukang Pharmaceuticals, Shandong, China) was used since the first day of treatment with an intravenous dosage of 5 mg/8 h for seven consecutive days, then 2.5 mg/12 h for 1 d, and stopped. If the patient defecated or passed gas in < 8 d after treatment, dexamethasone was withdrawn within 24 h after resolution of obstruction. During treatment, patients were carefully monitored for abdominal symptoms and systemic complications, such as cholestasis, central catheter infections, and systemic infections. Other complications, such as hypovolemia, electrolyte-fluid imbalance, and hyperglycemia, were corrected during treatment and not documented.

Indications for prompt surgery included suspicion for strangulation (continuous *vs* colicky pain, fever, tachycardia, peritoneal signs, and sustained leukocytosis), or clinical deterioration that implied failure of conservative management for > 3 mo.

The following parameters were recorded in each patient: age and sex; interval between symptom onset and the most recent laparotomy; clinical features including symptoms, presence or absence of fever, white blood cells, nutritional status, and comorbidity; procedures and duration of last operation; and time of previous laparotomy. Complete resolution of obstruction was established when symptoms and signs of obstruction subsided, normal flatus and defecation returned, and there was no relapse of obstructive symptoms after withdrawal of somatostatin. Then, liquid food or enteral nutrition was started. A semiliquid food was usually given 2 d later. Patients were discharged when intravenous fluid was stopped and semiliquid food was tolerated for 3 d.

Outcome measures

The primary endpoints of the study were time to resolution of obstruction and length of hospital stay, and the secondary endpoints were daily NG output, NG tube placement duration, treatment-related complications, postoperative obstruction relapse, and patient satisfaction.

Sample size calculation

Sample size calculation was based on our previous data of historical comparison[18], which showed a mean 26.0 d for the intervention group and 30.3 d for the control group, with mean ± SD of 9.0 d. Approximately 35 patients in each group were needed to detect a difference in hospital stay with 80% power and a two-sided 5% significance level.

Long-term follow up

The patients were followed for ⩾ 3 years after discharge. At each 6-mo visit, patients were given a questionnaire that was completed and returned by mail or they were contacted by telephone with the complete questions answered. Obstruction relapse was defined as abdominal pain with the halt of flatus, and nausea/vomiting, which needed further medical treatment and admission to hospital. At 1, 2 and 3 years, the degree of postoperative satisfaction was evaluated in every patient by using a unified scale (1-4) that indicated very unsatisfied, unsatisfied, satisfied, and very satisfied, respectively. Patient satisfaction was based on the core symptoms of the Gastrointestinal Quality of Life Index such as abdominal pain, feeling of abdominal distension, flatus and stool frequency, anorexia, fatigue, and nausea and vomiting[19]. The definition of "very satisfied" was the presence of none of the abovementioned gastrointestinal symptoms in the past year; "satisfied" was occasional gastrointestinal symptoms; "unsatisfied" was several episodes of abdominal symptoms in the past year, and "very unsatisfied" was frequent abdominal symptoms.

Statistical analysis

Statistical analysis was performed by per-protocol analysis. Quantitative variables, presented as mean ± SD (range), were analyzed by Mann-Whitney *U* test or Student's *t* test if appropriate. Quantitative variables, expressed as a number (percentage), were analyzed by Pearson's χ^2 test or Fisher's exact test. All analyses were performed with SPSS version 13.0 (SPSS, Chicago, IL, United States). $P < 0.05$ indicated statistical significance.

RESULTS

Patient disposition and baseline characteristics

Between June 2002 and January 2009, 82 patients were diagnosed with EPSBO-OP in our department. Six patients were aged < 18 years and two declined to participate in the study, which left 74 patients enrolled in the study. Two patients were eventually confirmed to have mechanical obstruction and two had intra-abdominal abscesses or anastomotic fistulae and withdrew from the study. The dropout patients were eventually proven not to have EPSBO-OP, therefore, we used per-protocol analysis instead of intention-to-treat statistical analysis. Therefore, 70 cases were evaluated (34 in the T group and 36 in the TDS group) (Figure 1).

Patients' demographic data and previous surgeries are listed in Tables 1 and 2. Fifteen patients (10 in the TDS group and 5 in the T group) had a history of malignancy but all underwent radical surgical resection. There were no significant differences between the two groups with respect to laboratory and clinical features at trial entry. The median onset of obstructive symptoms was postoperative day 9.4 ± 3.5 (range: 5-23 d).

Sixty-three patients underwent more than two operations before EPSBO-OP developed. At last surgery, extensive adhesiolysis (including intestinal splinting[20]) was performed in 54 (77.1%) of the operations; six (8.6%) received repeated laparotomy within 2 wk, and another six patients had diffuse peritonitis during last laparotomy. Although the mean number of operations (2.9 ± 1.3 *vs* 3.0 ± 1.0, $P = 0.927$) and type of operation were similar between the two groups, the operation time was shorter in the TDS group compared with the T group (4.1 ± 1.3 *vs*

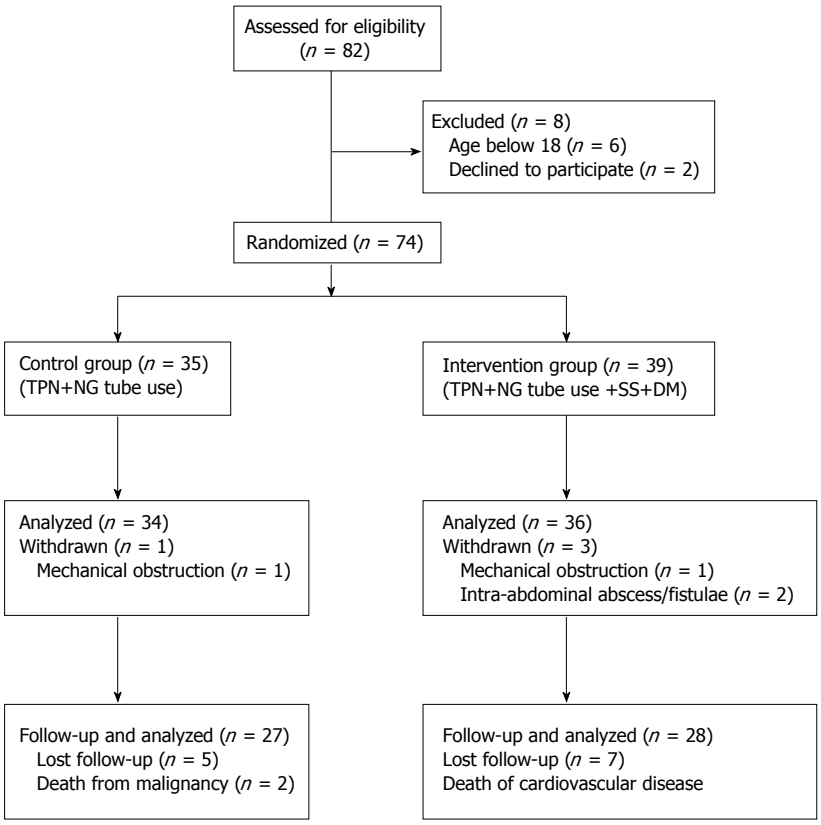

Figure 1 Flow chart of patient inclusion and follow-up. TPN: Total parenteral nutrition; NG: Nasogastric; DM: Dexamethasone; SS: Somatostatin.

Table 1 Demographic data and clinical features *n* (%)

	T group (*n* = 34)	TDS group (*n* = 36)	*P* value
Age (yr)	43.9 ± 10.2 (26-64)	45.4 ± 13.2 (20-78)	0.597
Sex ratio (M/F)	24/10	18/18	0.079
Symptoms onset			
≤ 1 POW	12 (35.3)	13 (36.1)	0.943
1-2 POW	19 (55.9)	21 (58.3)	0.836
2-3 POW	3 (8.8)	1 (2.8)	0.276
3-4 POW	0	1 (2.8)	
Mean POD of symptom onset	9.8 ± 3.4 (5-19)	9.1 ± 3.5 (5-23)	0.412
Symptoms			
Nausea and vomiting	32 (94.1)	33 (91.7)	0.691
Abdominal distension	23 (67.6)	20 (55.6)	0.299
Colic pain	0	0	
Obstipation	34 (100)	36 (100)	
Hyperthermia (> 37.5 °C)[1]	1 (2.9)	1 (2.6)	0.967
Maximum WBC (× 10^9/L)	7.0 ± 2.3 (3.7-12.3)	8.0 ± 3.0 (4.8-17.1)	0.148
Neutrophil (%)	71.2 ± 10.4 (48-88)	75.1 ± 8.4 (59-91)	0.084
Nutrition status on admission			
Mean BMI (kg/m^2)	20.4 ± 2.2 (16.8-26.4)	21.2 ± 2.8 (17.3-28.0)	0.248
Hypoalbuminemia (< 35g/L)	13 (38.2)	8 (22.2)	0.144
Anemia (Hb < 120g/L)	10 (29.4)	4 (11.1)	0.056
Comorbidity	3 (8.8)	4 (11.1)	0.750

[1]One due to pneumonia, and one due to epiglottis. POW: Postoperative week; POD: Postoperative day; BMI: Body mass index.

4.8 ± 1.1 h, $P = 0.041$). Typical radiographic and intraoperative findings at the last operation are shown in Figures 2 and 3, respectively.

Efficacy endpoints

Treatment was successful for all patients in both groups. The mean length of hospital stay was 30.5 ± 10.9 (16-69) d. No patients withdrew because they needed surgery for strangulation or failure of conservative therapy. There were no deaths during treatment. In the TDS group, the mean duration of somatostatin usage was 23.5 ± 9.1 (14-53) d, while dexamethasone was used for 8 d in all

Table 2 Previous laparotomies *n* (%)

	T group (*n* = 34)	TDS group (*n* = 36)	*P* value
No. of laparotomies			
1	3 (8.8)	4 (11.1)	0.750
2	7 (20.6)	11 (30.6)	0.340
3	15 (44.1)	11 (30.6)	0.241
4	6 (17.6)	4 (11.1)	0.435
⩾ 5	3 (8.8)	6 (16.7)	0.327
Mean No. (range)	3.0 ± 1.0 (1-5)	2.9 ± 1.3 (1-6)	0.927
Type of last operation			
Bowel obstruction	12 (35.3)	18 (50.0)	0.214
Enterocutaneous fistula	15 (44.1)	10 (27.8)	0.154
Enterectomy/colectomy	2 (5.9)	1 (2.8)	0.522
Gastroduodenal surgery	0	1 (2.8)	0.328
Hematoma removal	2 (5.9)	1 (2.8)	0.522
Appendectomy[1]	0	2 (5.6)	0.163
Laparotomy after trauma	1 (2.9)	1 (2.8)	0.967
Others	2 (5.9)	2 (5.6)	0.953
Patients with history of malignancy	5 (14.7)	10 (27.8)	0.183
At last operation			
with extensive enterolysis[2]	24 (70.6)	30 (83.4)	0.204
with diffuse peritonitis	4 (11.8)	2 (5.6)	0.354
< 2 wk from previous surgery	3 (8.8)	3 (8.3)	0.971
Last operation time (h)	4.8 ± 1.1 (2.0-6.5)	4.1 ± 1.3 (1.5-7)	0.041

[1]All are perforated appendicitis with diffuse peritonitis; [2]Including with intestinal splinting.

patients.

As shown in Table 3, somatostatin and dexamethasone had a marked effect on the recovery of bowel function, as indicated by earlier passage of stool or gas (22.4 ± 9.1 *vs* 29.9 ± 10.1 d, $P = 0.002$). The length of hospital stay in the intervention group was shorter than in the control group (25.8 ± 9.9 *vs* 34.7 ± 11.2 d, $P = 0.001$).

The daily NG output and NG duration were evaluated as indicators of symptom control. The daily NG output was 583 ± 208 (150-1050) mL in the TDS group, which was significantly lower ($P < 0.001$) than that in the T group [922 ± 399 (400-1825) mL]. The need for NG tube use was also significantly shorter in the TDS group (16.7 ± 8.8 *vs* 27.7 ± 9.9 d, $P < 0.001$).

Safety endpoints

Treatment-related complications are shown in Table 4. The rate of overall complications was comparable in the TDS and T group (41.7% *vs* 38.2%, $P = 0.770$). Cholestasis, as revealed by increased bilirubin, AKP, γ-glutamyltransferase, or biliary sludge on ultrasonography, developed in 13 patients, and percutaneous transhepatic cholecystostomy (PTC) was performed in eight patients presenting with acalculous cholecystitis. The incidence of cholestasis and need for PTC were higher in the TDS group, but not significantly ($P = 0.419$ and 0.264, respectively). Infectious complications, including catheter-related sepsis, wound infection, and pneumonia, occurred in 15 patients. All blood culture-positive, catheter-related sepsis was cured with antibiotics. Statistical analysis revealed that there was no significant difference in infectious complications between the two groups ($P = 0.677$). Two patients had pneumothorax on catheter insertion.

Treatment with somatostatin and dexamethasone was well tolerated and did not cause any serious or clinical significant adverse reactions except that one patient in the intervention group complained of dry mouth.

Follow-up outcomes

Twelve patients were lost to follow-up (7 in the TDS group and 5 in the T group), and two patients in the T group died of relapse of primary colon cancer and gastric cancer after 12 and 18 mo of follow-up, respectively, while one in the TDS group died of cardiovascular disease (at 30 mo follow-up). Long-term follow-up data indicated that the rates of recurrence of obstruction at 1, 2 and 3 years postoperatively were similar between the two groups (Table 3). In addition, there was no significant difference in postoperative satisfaction at 1, 2 and 3 years between the two groups (Table 3).

DISCUSSION

EPSBO-OP is a rare complication after major abdominal procedures, mostly after extensive adhesiolysis. Although conservative therapy was effective in most of our cases, the patients often had to be maintained on long-term NG suction and TPN therapy before recovery of bowel function. Our results suggested that dexamethasone and somatostatin, when added to TPN, decreased the duration of NG suction and daily NG output, and shortened the duration of bowel obstruction as well as the length of hospital stay.

EPSBO-OP was first described by Fazio *et al*[21] and Hill *et al*[22] in 1983. In contrast to the common causes of EPSBO such as local adhesion, volvulus, or internal hernia, which can be managed surgically after failure of conservative treatment[23,24], OP is caused by formation of dense adhesions and severe peritoneal reaction within the early postoperative period - typically 10 d to 6-8 wk after some major procedures - especially when the bowel has fistulated. The main risk factors include extensive adhesiolysis, multiple sequential laparotomies within a short period (*i.e.*, several days or weeks), peritonitis, and other factors causing extensive intestinal deserosalization[25,26]. The acute inflammatory reaction may involve the peritoneal surface and adherence of adjacent loops of bowel, often involving the omentum and mesenteric surfaces. These adhesions are highly vascularized, friable, and immature, thus, surgical separation is impossible. Therefore, recognition of EPSBO-OP is important to avoid serious consequences such as ECF or massive bowel resection because of re-laparotomy attempting to lyse the adhesions[27]. The adhesions are extensive, thus, the risk of closed-loop obstruction, volvulus, or strangulation is low, making conservative therapy possible[4].

Resolution of OP after prolonged TPN therapy has been reported previously. Lennard *et al*[8] reported two patients with ECF and OP managed with TPN for 8

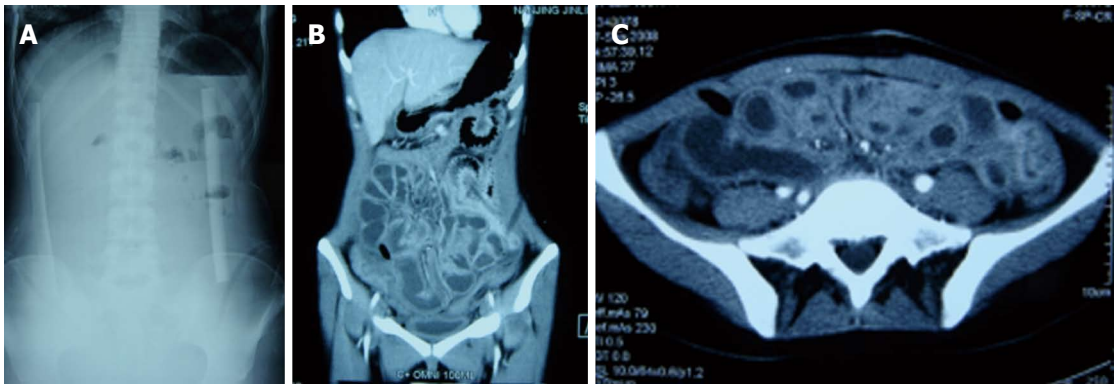

Figure 2 Typical radiographic and intra-operative finding in the last operation. A: Typical upright radiograph of early postoperative small bowel obstruction with obliterative peritonitis after extensive adhesiolysis for abdominal cocoon, showing only mild air-fluid levels. No isolated small bowel loops were observed; B, C: Computed tomography scan reveals edematous small bowel filled-up with fluids. The border between the small bowel loops is not clear. No significant discrepancies in small bowel diameter and air-fluid levels were observed.

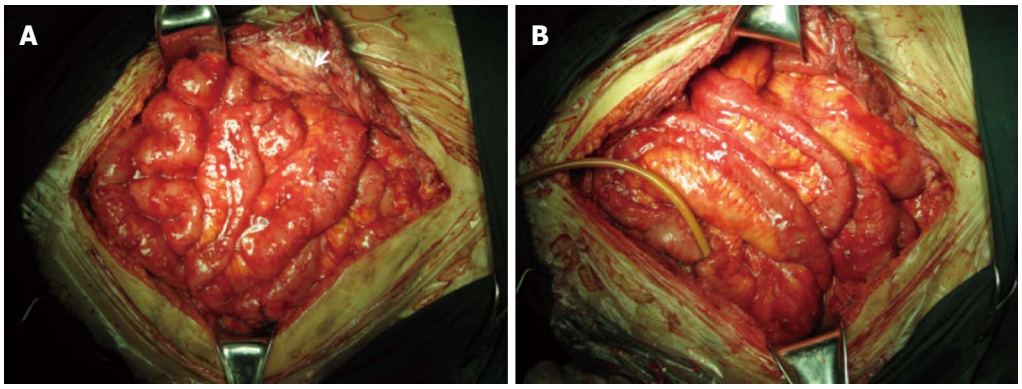

Figure 3 Intraoperative photo of a patient who developed early postoperative small bowel obstruction with obliterative peritonitis. The 40-year-old male had a colostomy and patch-repair (arrow) of the abdominal defect after open abdomen due to trauma. He required closure of the stoma 6 months after colostomy. On operation, dense matted adhesions were found, especially beneath the patch, and full enterolysis resulted in extensive intestinal deserosalization (A). An intestinal splinting was performed (B). On postoperative day (POD) 10 the tube was removed, and he had a temporary return of bowel function but early postoperative small bowel obstruction with obliterative peritonitis on POD 15.

and 4 mo, respectively. Selby *et al*[9] reported six patients with EPSBO secondary to dense and vascular benign adhesions that could not be freed by operation. The obstructions all spontaneously resolved within 2-3.5 mo on a TPN program. The authors believe that complete gastrointestinal rest allows adhesions to mature into long avascular collagen fibers in the absence of a persistent inflammatory reaction that accompanies partial or total SBO. In the current study, we also confirmed that with TPN alone, EPSBO-OP could also resolve, but it often takes a long time.

Somatostatin, or its synthetic analog octreotide, inhibits gastrointestinal secretion and release of hormones, and they have been used for treatment of SBO for over a decade, especially for malignant SBO[28]. In experimental animal models, somatostatin may be beneficial for the control of intestinal distension, inflammation and necrosis, and bacterial translocation[29,30]. Octreotide, the somatostatin analog, may ameliorate intestinal dysmotility and stasis in models of small bowel transplantation[31]. Besides its role in symptom control, somatostatin may also promote the resolution of SBO[32,33]. Zhang *et al*[34] have confirmed that octreotide, when combined with water-soluble radiocontrast medium, may accelerate resolution of adhesive SBO by a specific therapeutic affect. This is consistent with the findings of the current study.

Corticosteroids have long been used for their anti-inflammatory effects, which may reduce the edema and fibrin deposition associated with EPSBO-OP, thereby helping to resolve the obstruction[35]. In Japan, steroids have been used to reduce the inflammatory state of encapsulating peritoneal sclerosis, in which intraperitoneal inflammation leads to adhesive and inflammatory encapsulation of the intestinal tract, causing bowel obstructive symptoms. In a prospective cohort, 15 of 42 cases (35.7%) of encapsulating peritoneal sclerosis treated with prednisolone alone showed clinical improvement[14]. In malignant bowel obstruction, corticosteroids may reduce intestinal inflammatory edema associated with the malignant lesion, thereby aiding resolution of bowel obstruction[14]. Extensive dense inflammatory adhesions and intestinal wall edema are characteristics of EPSBO-OP, therefore, we explored the effect of corticosteroids in EPSBO-OP, and our data showed that DM, when combined with so-

Table 3 Outcomes of patients receiving total parenteral nutrition alone or in combination with somatostatin and dexamethasone

	TPN (n = 34)	TDS (n = 36)	P value
Time to obstruction resolution (d)	29.9 ± 10.1 (17-60)	22.4 ± 9.1 (13-52)	0.002
Length of hospital stay (d)	34.7 ± 11.2 (21-69)	25.8 ± 9.9 (16-57)	0.001
Daily NG output (mL)[1]	922 ± 399 (400-1825)	583 ± 208 (150-1050)	< 0.001
Mean NG duration (d)	27.7 ± 9.9 (7-54)	16.7 ± 8.8 (3-42)	< 0.001
Relapse of obstruction			
1 yr after operation (n/N)[2]	3/32	2/34	0.668
2 yr after operation (n/N)[3]	6/29	5/31	0.745
3 yr after operation (n/N)[4]	8/27	6/28	0.547
Postoperative satisfaction ≥ 3[5]			
1 yr after operation (n/N)[2]	22/32	20/34	0.451
2 yr after operation (n/N)[3]	15/29	14/31	0.796
3 yr after operation (n/N)[4]	11/27	10/28	0.785

[1]Mean value of the first 2 d after NG tube placement; [2]TPN (n = 32, 1 patient was lost to follow-up, and 1 died of malignancy); TDS (n = 34, 2 patients were lost to follow-up); [3]TPN (n = 29, 2 patients were lost to follow-up, and 1 died of malignancy); TDS (n = 31, 3 patients were lost to follow-up); [4]TPN (n = 27, 2 patients were lost to follow-up); TDS (n = 28, 2 patients were lost to follow-up and 1 died of cardiovascular disease); [5]Patients with satisfaction ≥ 3 were satisfied (3) or very satisfied (4). TDS: TPN + DM + SS; TPN: Total parenteral nutrition; DM: Dexamethasone; SS: Somatostatin; NG: Nasogastric.

Table 4 Treatment-related complications n (%)

	TPN (n = 34)	TDS (n = 36)	P value
Morbidity			
Cholestasis	5 (14.7)	8 (22.2)	0.419
Patients requiring PTC	2 (5.9)	5 (13.9)	0.264
Infectious complications	8 (23.5)	7 (19.4)	0.677
Catheter-related infections	5 (14.7)	4 (11.1)	0.653
Wound infection	2 (5.9)	1 (2.8)	0.522
Pneumonia	1 (2.9)[1]	0	0.300
Pneumothorax	0	2 (5.5)	0.163
Overall complications	13 (38.2)	15 (41.7)	0.770

[1]Patient had tracheostomy. TDS: TPN + DM + SS; TPN: Total parenteral nutrition; DM: Dexamethasone; SS: Somatostatin; PTC: Percutaneous transhepatic cholecystostomy.

matostatin, promoted resolution of the adhesions. Fibrin exudation and intestinal edema were most prominent in the early stage of EPSBO-OP, thus, we recommend the usage of DM immediately after diagnosis.

Cholestasis is a complication of long-term usage of somatostatin[36] and TPN. Animal models and human volunteer studies all suggest that the effect of somatostatin is associated with a pronounced decrease in bile flow, bile acid secretion, and increased bile cholesterol saturation[37-39]. In the current study, although we observed an increased incidence of cholestasis and need for PTC in patients receiving somatostatin, it did not reach statistical significance. This was possibly due to the small number of cases in our series. However, it could also be that the beneficial effect of dexamethasone on bile excretion partly counteracts the detrimental effect of somatostatin[40,41].

Increased susceptibility to infection and impaired wound healing are the main side effects of systemic corticosteroids. Trésallet *et al*[42] have observed that patients on steroids for > 1 mo had a higher incidence of postoperative complications, especially infections after colectomy with rectal anastomosis. We did not observe any difference in the occurrence of infection between the two groups, which was possibly because we used short-term therapy (7 d). There is currently no direct evidence that dexamethasone promotes relapse of malignancy, therefore, we did not avoid its use in tumor patients.

There were several limitations to our study. First, the diagnosis of OP was made on the basis of clinical presentation, physical examination, and medical history, and was confirmed by plain film radiography and CT, but it could not be definitively proven by laparotomy. Therefore, this may have led to the inclusion of a few cases of obstruction not caused by OP. Second, the mean operation time preceding obstruction was shorter in the TDS group, and this may have influenced the outcome. Third, the study was not blinded and the physicians and patients were aware of which therapy that each patient had received, which would have introduced some bias during evaluation.

In conclusion, our trial indicates that conservative therapy is efficient in EPSBO-OP. Administration of somatostatin and dexamethasone in addition to TPN promotes resolution of obstruction, shortens length of hospital stay, and is efficient for symptom control without increasing complications and obstruction relapse.

COMMENTS

Background

Early postoperative small bowel obstruction due to obliterative peritonitis (EPSBO-OP) is a rare complication after abdominal surgery, especially extensive adhesiolysis and enterocutaneous fistula. Traditionally, the only treatment for these patients was total parenteral nutrition, nasogastric tube feeding, and observation. The time to the recovery of bowel function is often long and patients often suffer from low quality of life. Methods to promote resolution and control obstruction-related symptoms are lacking.

Research frontiers

Somatostatin or its analogs and corticosteroids are effective and safe in patients with inoperative bowel obstruction due to peritoneal carcinomatosis or encapsulating peritoneal sclerosis. Therefore, their clinical role in the management of postoperative OP warrants further investigation.

Innovations and breakthroughs

Somatostatin and dexamethasone shorten the time to obstruction resolution and length of hospital stay, and decrease nasogastric output and duration of

nasogastric tube usage. They do not increase treatment-related complications and relapse of obstruction.

Applications

This study revealed that somatostatin and dexamethasone are effective in promoting resolution and controlling symptoms in patients with EPSBO-OP.

Peer review

This paper provides some useful information on the management of EPSBO-OP.

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BRIEF ARTICLE

Preoperative biliary drainage in patients with hilar cholangiocarcinoma undergoing major hepatectomy

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Abstract

AIM: To investigate the effect of preoperative biliary drainage (PBD) in jaundiced patients with hilar cholangiocarcinoma (HCCA) undergoing major liver resections.

METHODS: An observational study was carried out by reviewing a prospectively maintained database of HCCA patients who underwent major liver resection for curative therapy from January 2002 to December 2012. Patients were divided into two groups based on whether PBD was performed: a drained group and an undrained group. Patient baseline characteristics, preoperative factors, perioperative and short-term postoperative outcomes were compared between the two groups. Risk factors for postoperative complications were also analyzed by logistic regression test with calculating OR and 95%CI.

RESULTS: In total, 78 jaundiced patients with HCCA underwent major liver resection: 32 had PBD prior to operation while 46 did not have PBD. The two groups were comparable with respect to age, sex, body mass index and co-morbidities. Furthermore, there was no significant difference in the total bilirubin (TBIL) levels between the drained group and the undrained group at admission (294.2 ± 135.7 *vs* 254.0 ± 63.5, $P = 0.126$). PBD significantly improved liver function, reducing not only the bilirubin levels but also other liver enzymes. The preoperative TBIL level was significantly lower in the drained group as compared to the undrained group (108.1 ± 60.6 *vs* 265.7 ± 69.1, $P = 0.000$). The rate of overall postoperative complications (53.1% *vs* 58.7%, $P = 0.626$), reoperation rate (6.3% *vs* 6.5%, $P = 1.000$), postoperative hospital stay (16.5 *vs* 15.0, $P = 0.221$) and mortality (9.4% *vs* 4.3%, $P = 0.673$) were similar between the two groups. In addition, there was no significant difference in infectious complications (40.6% *vs* 23.9%, $P = 0.116$) and noninfectious complications (31.3% *vs* 47.8%, $P = 0.143$) between the two groups. Univariate and multivariate analyses revealed that preoperative TBIL > 170 μmol/L (OR = 13.690, 95%CI: 1.275-147.028, $P = 0.031$), Bismuth-Corlette classification (OR = 0.013, 95%CI: 0.001-0.166, $P = 0.001$) and extended liver resection (OR = 14.010, 95%CI: 1.130-173.646, $P = 0.040$) were independent risk factors for postoperative complications.

CONCLUSION: Overall postoperative morbidity and mortality rates after major liver resection are not improved by PBD in HCCA patients with jaundice. Preoperative TBIL > 170 μmol/L, Bismuth-Corlette classification and extended liver resection are independent risk

factors linked to postoperative complications.

Key words: Obstructive jaundice; Hilar cholangiocarcinoma; Preoperative biliary drainage; Major hepatectomy; Surgical outcome

Core tip: There is currently no consensus on the use of preoperative biliary drainage (PBD) in jaundiced patients with hilar cholangiocarcinoma undergoing major liver resection. We retrospectively analyzed prospectively maintained database of these patients who underwent PBD or not. The baseline characteristics, perioperative and short-term postoperative outcomes between these two groups were compared and no significant differences were identified. We found that a preoperative total bilirubin level > 170 μmol/L, Bismuth-Corlette classification and extended liver resection are three independent risk factors for postoperative complications. There is a need to undertake well-designed, prospective multicenter studies to inform future practice.

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INTRODUCTION

Hilar cholangiocarcinoma (HCCA), which was first defined by Klatskin[1] as an adenocarcinoma of the hepatic duct at its bifurcation within the porta hepatis, is associated with a poor prognosis[1,2]. Currently, the only curative treatment is radical surgical resection[3]. However, a R0 resection margin is difficult to achieve because the tumor often infiltrates the portal vein, the hepatic artery and liver parenchyma[4,5]. In order to obtain negative histological margins and improve survival, many surgeons have adopted a more aggressive surgical approach, namely, extended hepatectomy combined with portal vein or hepatic artery resection and reconstruction, and hepato pancreaticoduodenectomy for the treatment of this malignancy[6-8]. However, the majority of patients with HCCA have obstructive jaundice at presentation, which increases the risk of complications, such as sepsis, bleeding and liver failure, especially in patients undergoing major hepatectomy[9,10]. Therefore, preoperative biliary drainage (PBD) was introduced with the aim to abrogate these potential complications in patients with jaundice secondary to HCCA, despite that a consensus on an appropriate cut-off level of total bilirubin (TBIL)[11-14] and duration of drainage[8,15,16] has not been reached yet.

There is still controversy with regard to whether PBD is essentially needed for jaundiced patients with HCCA undergoing major liver resection. It was shown that PBD reverses cholestasis-associated hepatic and systemic toxicity, and improves liver function, nutritional status and cell-mediated immune function[17]. However, concerns were also raised as PBD may associate with an increased incidence of postoperative morbidity and mortality[18-20], although this was not the case for other studies[21,22]. Recently, one multicenter European study including patients undergoing major liver resection for HCCA suggested that overall morbidity was not affected by PBD procedure[14]. Furthermore, preoperative portal vein embolization (PVE), which is restricted to the treatment of postoperative inadequate residual liver volume and induces hypertrophy of the future remnant liver, has led to a change to PBD strategy[23]. PBD followed by PVE prior to major hepatectomy is considered a safe management strategy for HCCA, particularly in patients with remnant liver volume less than 40%[24-26].

The aim of this study was to inform the debate by comparing the perioperative and short-term postoperative outcomes of jaundiced patients with HCCA undergoing curative major liver resection with or without PBD, at a large specialist center in China.

MATERIALS AND METHODS

Study population and preoperative management

The prospectively maintained database for a cohort of consecutive HCCA patients treated at the West China Hospital of Sichuan University between January 2002 and December 2012 was retrospectively reviewed. From the database, only patients with HCCA who had jaundice and underwent major hepatectomy for curative resection were included in this study. Jaundice was defined as a serum TBIL level > 85.5 μmol/L (5 mg/dL). HCCA was defined as lesions arising from the common hepatic duct, left, right, or both hepatic duct and intrahepatic bile duct cancer invading the hepatic hilus[11]. The tumors were classified according to Bismuth-Corlette classification[27].

In our series, blood sampling for serum biochemistry was completed 2-3 d before drainage or surgery. Color Doppler ultrasound and contrast enhanced computed tomography (CT) were used routinely before surgery. Furthermore, magnetic resonance imaging (MRI) was used in most of patients. If distant metastases were suspected, further investigations with positron emission tomography-CT scan were performed. PVE was carried out at our hospital if the remnant liver volume post surgical resection was expected to be less than 50% of the whole liver volume. PBD was performed if patients fulfilled one of the following criteria: duration of jaundice of more than 4 wk; poor nutritional status (serum albumin < 3 g/dL); signs of cholangitis. PBD procedures in our center were percutaneous transhepatic cholangio-drainage (PTCD), endoscopic biliary stenting (EBS), endoscopic nasobiliary drainage (ENBD) and/or surgical drainage. For patients who had inadequate PBD before admission to our hospital, a further drainage by a percutaneous approach was

adapted. Adequate PBD was evident by a relief of cholangitis, and an improvement in the liver function and/or the nutritional status of the patient.

Surgical procedures

At our center, curative excision was defined as histologically negative surgical margins with a minimum tumor-free margin of 5 mm at the hepatic stump of the bile duct, the duodenal stump of the bile duct, and the excision surface. It included resection of the gallbladder and extrahepatic bile duct; skeletonization of the vasculature of the hepatoduodenal ligament; and partial hepatectomy, or even removal of the caudate lobe or portal vein or hepatic artery as required. The postoperative biliary drainage was established by a Roux-en-Y hepaticojejunostomy. Major hepatectomy was defined as resection of three or more Couinaud segments. Caudate lobectomy was performed in patients in whom it was considered necessary to achieve complete tumor clearance.

Postoperative complications

While patients were followed routinely after discharge from hospital, as part of this study, we endeavored to investigate the effect of PBD on in-hospital postoperative outcomes. Hence, postoperative mortality was defined as death prior to hospital discharge. All postoperative complications were defined as events that lengthened hospital stay. Infectious complications were defined according to the study by Hochwald *et al*[19]; these were intraabdominal abscess, wound infection, cholangitis, sepsis and lung infection. Noninfectious complications included liver failure, bile leak, anastomotic leak, abdominal collection, gastrointestinal bleeding, abdominal bleeding, respiratory failure and renal failure. Liver failure was defined as an increased international normalized ratio and concomitant hyperbilirubinemia on or after postoperative day five[28]. Bile leak was defined as the drainage of 50 mL or more of bile from the surgical drain or from drainage of an abdominal collection, over a period of three days or more[29]. In addition, the complications were graded according to the Clavien-Dindo classification of surgical complications[30].

Literature search

Existing literature was also reviewed by performing a systematic search in PubMed, Medline and Embase from January 1990 to May 2013. The following search terms were used: "preoperative biliary drainage" or "percutaneous transhepatic biliary drainage" or "endoscopic biliary drainage" or "endoscopic nasobiliary drainage" or "endoscopic biliary stenting" and "hilar cholangiocarcinoma" or "hilar bile duct cancer" or "proximal bile duct cancer" or "Klatskin tumor" or "carcinoma of the hepatic duct confluence" along with their synonyms or abbreviations. The search was restricted to studies conducted on human subjects and in the English language only.

Statistical analysis

Data are presented as mean ± SD or median and interquartile range. The χ^2 test or Fisher's exact test or RxC table analysis was used to compare categorical variables, and the Student's *t* test or Mann-Whitney *U* test was used to compare continuous variables. A statistically significant difference was defined as a P value < 0.05. The variables of statistical significance during univariate analysis were included in a follow-up multivariate analysis, by using the logistic regression test. The OR and 95%CI were also calculated for individual factors in the multivariate analysis. All statistical analyses were performed with SPSS software (SPSS version 17.0, Chicago, Illinois).

Table 1 Baseline characteristics *n* (%)

	Drained (*n* = 32)	Undrained (*n* = 46)	*P* value
Age (yr)	59.6 ± 11.0	58.2 ± 11.3	0.568
Sex (M/F)	21/11	28/18	0.669
Body mass index (kg/m^2)	20.3 ± 1.9	21.0 ± 2.5	0.190
Concomitant diseases			
Diabetes	2 (6.3)	3 (6.5)	1.000
Hypertension	3 (9.4)	7 (15.2)	0.678
Cardiovascular	2 (6.3)	5 (10.9)	0.765
Previous history of abdominal surgery	9 (28.1)	12 (26.1)	0.842
Serum total bilirubin (μmol/L)			
At admission	294.2 ± 135.7	254.0 ± 63.5	0.126
Before surgery	108.1 ± 60.6	265.7 ± 69.1	0.000
Time of PBD (d)	15.3 ± 3.4	-	-
Time between admission and surgery (d)	20.7 ± 2.1	3.8 ± 1.6	0.000
Portal vein embolization	5 (15.6)	3 (6.5)	0.355
Bismuth–Corlette classification			
Ⅰ	1 (3.1)	1 (2.2)	1.000
Ⅱ	8 (25)	14 (30.4)	0.600
Ⅲa	6 (18.8)	7 (15.2)	0.680
Ⅲb	9 (28.1)	15 (32.6)	0.673
Ⅳ	8 (25)	9 (19.6)	0.567
Perioperative details			
Hilar bile duct resection	32 (100)	46 (100)	-
Left hepatectomy	17 (53.1)	31 (67.4)	0.203
Extended left hepatectomy	2 (6.3)	1 (2.2)	0.747
Right hepatectomy	8 (25)	10 (21.7)	0.737
Extended right	5 (15.6)	4 (8.7)	0.561
Hepatectomy			
Caudate lobectomy	8 (25)	12 (26.1)	0.914
Pedicle clamping	17 (53.1)	26 (56.5)	0.767
Portal vein resection	6 (18.8)	8 (17.4)	0.878
Hepatic artery resection	2 (6.3)	3 (6.5)	1.000
Number of blood	11 (34.4)	24 (52.2)	0.120
Transfusions			
Intraoperative blood transfusion (mL)	900 (800-900)	800 (600-1100)	0.513

PBD: Preoperative biliary drainage.

RESULTS

Baseline characteristics

During the study period, 78 patients with jaundice underwent major hepatic resection for HCCA at our hospital. There were 32 patients in the drained (PBD) group and 46 patients in the undrained (no PBD) group. The baseline characteristics of patients are outlined in Table 1. The drained group was comparable with the undrained

Table 2 Postoperative outcomes of patients undergoing major hepatectomy *n* (%)

	Drained (*n* = 32)	Undrained (*n* = 46)	*P* value
Morbidity	17 (53.1)	27 (58.7)	0.626
Infectious morbidity	13 (40.6)	11 (23.9)	0.116
Intra-abdominal abscess (Ⅱ-Ⅲa)	3 (9.4)	2 (4.3)	0.673
Wound infection (Ⅰ-Ⅲb)	4 (12.5)	4 (8.7)	0.869
Cholangitis (Ⅱ)	1 (3.1)	2 (4.3)	1.000
Sepsis (Ⅳa-Ⅴ)	2 (6.3)	1 (2.2)	0.747
Lung infection (Ⅱ)	6 (18.8)	5 (10.9)	0.325
Noninfectious morbidity	10 (31.3)	22 (47.8)	0.143
Liver failure (Ⅱ-Ⅴ)	3 (9.4)	6 (13)	0.890
Bile leak			
Remnant liver[1] (Ⅱ-Ⅲa)	2 (6.3)	4 (8.7)	1.000
Anastomotic leak[2] (Ⅱ-Ⅲb)	1 (3.1)	2 (4.3)	1.000
Abdominal collection (Ⅰ-Ⅲa)	6 (18.8)	9 (19.6)	0.928
Gastrointestinal bleeding (Ⅲa-Ⅴ)	0	2 (4.3)	0.510
Abdominal bleeding (Ⅱ-Ⅲb)	1 (3.1)	2 (4.3)	1.000
Respiratory failure (Ⅳa)	0	3 (6.5)	0.265
Renal failure (Ⅳa-Ⅴ)	3 (9.4)	4 (8.7)	1.000
Mortality (Ⅴ)	3 (9.4)	2 (4.3)	0.673
Reoperation	2 (6.3)	3 (6.5)	1.000
Postoperative hospital stay (d)	16.5 (13.5-20.5)	15 (12-18)	0.221

Clavien-Dindo grades of surgical complications are within parentheses. [1]From remnant liver; [2]From hepaticojejunostomy.

group with regards to age, sex, body mass index, comorbidity and previous history of abdominal surgery (P > 0.05 for all). Nine patients in the PBD group had previous abdominal surgery that included 5 cholecystectomies and 4 common bile duct explorations with T-tube drainage. Twelve patients in the undrained group had previous abdominal surgery, which included 6 appendectomies, 4 cholecystectomies and 2 cholecystectomies with common bile duct exploration. Furthermore, there was no significant difference in the TBIL levels at admission between the drained and undrained groups (294.2 ± 135.7 *vs* 254.0 ± 63.5, P = 0.126).

PBD techniques and liver function tests

In the PBD group, 23, 5 and 4 patients underwent PTCD, ENBD, and surgical drainage, respectively. In this study, 4 patients underwent surgical drainage through laparotomy and T-tube placement at the referring hospitals. No patient in this study underwent EBS. Six patients underwent PTCD twice each as a result of previous inadequate drainage. Drainage-related complications occurred in 8 patients (10.3%), with 3 cases of cholangitis and 4 of hemobilia following PTCD, and 1 case of hyperamylasemia following ENBD. All these adverse events were resolved after symptomatic treatment alone before surgery. The mean time between insertion of a biliary drainage catheter preoperatively and surgical resection was 15.3 ± 3.4 (d). PBD significantly improved liver function as evidenced by reduced TBIL (294.2 ± 135.7 *vs* 108.1 ± 60.6, P = 0.000), direct bilirubin (DBIL) (231.8 ± 87.0 *vs* 85.2 ± 57.4, P = 0.000), aspartate aminotransferase (AST) (132.1 ± 68.6 *vs* 86.1 ± 35.8, P = 0.000), alanine aminotransferase (ALT) (123.2 ± 79.1 *vs* 97.5 ± 62.4, P = 0.004), gamma-glutamyl transpeptidase (GGT) (531.2 ± 434.7 *vs* 357.6 ± 268.3, P = 0.000) and alkaline phosphatase (ALP) (502.1 ± 356.2 *vs* 343.5 ± 187.6, P = 0.001), although albumin (ALB) (36.7 ± 4.8 *vs* 34.8 ± 5.9, P = 0.213) levels remained unchanged.

Perioperative details

All patients in both groups had hilar bile duct resection. There were no significant differences in operation procedure (liver resection) between the two groups. Also, there were no significant differences between the drained and undrained groups in terms of caudate lobectomy, pedicle clamping, portal vein resection, hepatic artery resection, number of patients requiring blood transfusions and intraoperative blood transfusion volume (all P > 0.05).

Postoperative outcomes

Postoperative outcomes are outlined in Table 2. The number of patients with postoperative morbidity in the two groups was comparable (53.1% *vs* 58.7%, P = 0.626). No significant difference was found in the number of patients who had either infectious morbidity or non-infectious morbidity. Also, there was no significant difference in the incidence of individual complications. In addition, in a subgroup analysis (data not shown in table), there was a higher morbidity (84.6% *vs* 35.7%, P = 0.028) in patients undergoing right-sided hepatectomy without PBD than patients with PBD. However, in the left-sided hepatectomy group, patients had a higher morbidity (78.9% *vs* 40.6%, P = 0.018) in the drained group compared to the undrained group. However, there was no difference in the postoperative hospital stay between the two groups (16.5 *vs* 15, P = 0.221). Two patients in the drained group and 3 patients in the undrained group underwent reoperation. There was no significant difference in mortality (9.4% *vs* 4.3%, P = 0.673) between the two groups. In the drained group, 1 patient died of multiorgan failure (liver failure and renal failure) while another 2 patients died of septic shock. In the undrained group, one patient died from a massive gastrointestinal bleeding while another 1 patient died of multiorgan failure (liver failure and renal failure).

Logistic regression analyses

Several variables in this study were analyzed for their association with postoperative morbidity (Table 3). Univariate logistic regression showed that PBD was not a risk factor associated with postoperative morbidity. However, preoperative TBIL > 170 μmol/L (P = 0.021), preoperative AST > 100 U/L (P = 0.036), Bismuth-Corlette classification (P = 0.025) and extended liver resection (P = 0.018) were risk factors associated with postoperative morbidity on univariate logistic regression analysis. Furthermore, multivariate analysis identified preoperative TBIL > 170 μmol/L (OR = 13.690, 95%CI: 1.275-147.028, P = 0.031), Bismuth-Corlette classification (OR = 0.013, 95%CI: 0.001-0.166, P = 0.001) and extended liver resection (OR = 14.010, 95%CI: 1.130-173.646, P = 0.040) as three independent risk fac-

Table 3 The risk factors for postoperative complications *n* (%)

Variable	*n*	Incidence of complications	Univariate	Multivariate	
			P value	OR	*P* value
Age (yr)					
> 60	38	21 (55.3)	0.842		
≤ 60	40	23 (57.5)			
Sex					
Male	49	25 (51)	0.212		
Female	29	19 (65.5)			
PBD					
Yes	32	17 (53.1)	0.626		
No	46	27 (58.7)			
Concomitant diseases					
Yes	20	14 (70)	0.155		
No	58	30 (51.7)			
Previous abdominal surgery					
Yes	21	11 (52.4)	0.663		
No	57	33 (57.9)			
Preoperative TBIL					
> 170 μmol/L	48	32 (66.7)	0.021	13.690 (1.275-147.028)	0.031
≤ 170 μmol/L	30	12 (40)			
Preoperative AST					
> 100 U/L	47	31 (66)	0.036	1.138 (0.157-8.225)	0.898
≤ 100 U/L	31	13 (41.9)			
Preoperative ALT					
> 100 U/L	44	29 (65.9)	0.054	5.664 (0.595-53.905)	0.131
≤ 100 U/L	34	15 (44.1)			
Preoperative ALB					
> 35	41	26 (63.4)	0.189		
≤ 35	37	18 (48.6)			
Bismuth-Corlette stage					
Ⅰ and Ⅱ	24	9 (37.5)	0.025	0.013 (0.001-0.166)	0.001
Ⅲ and Ⅳ	54	35 (64.8)			
Extended liver resection					
Yes	12	11 (91.7)	0.018	14.010 (1.130-173.646)	0.04
No	66	33 (50)			
Caudate lobectomy					
Yes	20	12 (60)	0.707		
No	58	32 (55.2)			
Pedicle clamping					
Yes	43	22 (51.2)	0.300		
No	35	22 (62.9)			
Vascular resections					
Yes	19	13 (68.4)	0.225		
No	59	31 (52.5)			
Additional surgery					
Yes	6	3 (50)	0.742		
No	72	41 (56.9)			
Intraoperative blood transfusion					
Yes	35	19 (54.3)	0.644		
No	43	25 (59.5)			

TBIL: Total bilirubin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALB: Albumin; PBD: Preoperative biliary drainage.

tors for postoperative complications.

Results of literature search

Fourteen studies were identified[5,10,14,18-20,31-37] using the defined search strategy (Table 4). Seven studies included patients who had curative resections only[5,14,18,20,31,33,37], while the remaining studies included both curative and palliative resection groups.

DISCUSSION

Currently, the only curative treatment for HCCA is radical surgical resection[3]. Patients with HCCA usually present with concomitant obstructive jaundice, which results in high surgical morbidity and mortality in those undergoing major hepatic resection[38,39]. Furthermore, postoperative liver failure is a common cause of in-hospital death after major hepatectomy in patients with obstructive jaundice[13,40]. PBD offers the advantage of being able to increase the tolerance of cholestatic liver to ischemia, improve the regeneration capacity of the liver and decrease blood loss, which may contribute to reducing morbidity and mortality. However, there were conflicting conclusions from various studies with regards to the benefits of

Table 4 Studies including resections for hilar cholangiocarcinoma with and without preoperative biliary drainage *n* (%)

Ref.	Year	Country	Design	Type of PBD	Surgical procedures for included patients	PBD	*n*	Morbidity	*P* value	Mortality	*P* value
Su *et al*[10]	1996	China	Retro	PTCD	CR and PR	Yes	33	17 (51.5)	NS	5 (15.2)	NS
						No	16	6 (37.5)		0	
Takada *et al*[37]	1996	Japan	Retro	PTCD	CR	Yes	24	NA	-	3 (12.5)	NS
						No	12	NA		6 (50)	
Hochwald *et al*[19]	1999	United States	Pro	PTCD EBD	CR and PR	Yes	42	36 (85.7)	0.045	2 (4.8)	NS
						No	29	19 (65.5)		4 (14.3)	
Figueras *et al*[31]	2000	Spain	Retro	PTCD	CR	Yes	11	11 (100)	NS	1 (9)	NS
						No	9	6 (66)		2 (22.2)	
Parks *et al*[36]	2000	United Kingdom	Retro	PTCD EBD	CR and PR	Yes	20	11 (55)	NS	1 (5)	NS
						No	27	11 (40.7)		1 (3.7)	
Gerhards *et al*[5]	2000	The Netherlands	Retro	PTCD EBD	CR	Yes	93	59 (63)	NS	16 (17)	NS
						No	18	13 (72)		3 (17)	
Dinant *et al*[33]	2006	The Netherlands	Retro	PTCD EBD	CR	Yes	83	56 (67.5)	NS	14 (16.7)	NS
						No	14	6 (42.9)		2 (14.3)	
Li *et al*[32]	2009	China	Retro	PTCD	CR and PR	Yes	55	20 (36.3)	NS	4 (7.3)	NS
						No	56	16 (28.6)		5 (8.9)	
Ferrero *et al*[18]	2009	Italy	Retro	PTCD EBD SD	CR	Yes	30	21 (70)	NS	1 (3)	NS
						No	30	19 (63)		3 (10)	
Ercolani *et al*[34]	2010	Italy	Retro	PTCD EBD	CR and PR	Yes	44	25 (56.8)	NS	NA	-
						No	7	2 (28.5)		NA	
El-Hanafy *et al*[20]	2010	Egypt	Retro	PTCD EBD	CR	Yes	46	27 (58.6)	0.001	5 (10.8)	NS
						No	54	11 (20.3)		3 (5.5)	
Yu *et al*[35]	2012	China	Retro	PTCD with bile re-infusion	CR and PR	Yes	48	14 (29.2)	0.036	1 (2.1)	NS
						No	39	20 (51.3)		2 (5.1)	
Farges *et al*[14]	2013	France and Belgium	Retro	PTCD EBD	CR	Yes	180	123 (68.3)	NS	17 (9.4)	NS
						No	186	128 (68.8)		22 (11.8)	
Present study		China	Retro	PTCD EBD SD	CR	Yes	32	17 (53.1)	NS	3 (9.4)	NS
						No	46	27 (58.7)		2 (4.3)	

Retro: Retrospective; Pro: Prospective; PBD: Preoperative biliary drainage; PTCD: Percutaneous transhepatic cholangio-drainage; EBD: Endoscopic biliary drainage; SD: Surgical drainage; CR: Curative resection; PR: Palliative resection; NA: Not available; NS: Not significant.

PBD[14,19,20,31,35,41].

In our study, the two groups were comparable with respect to demographics, BMI, comorbidities and serum TBIL levels at admission. PBD-associated complications were low, occurring in 8 patients (10.3%), which may be because EBS was not used in our study[42]. This also illustrates that the drainage techniques and technology used at our center are feasible. The number of patients undergoing PVE before surgery was comparable between the drained and undrained groups. PBD followed by PVE prior to major hepatectomy is considered a safe management strategy[24-26].

The number of patients with postoperative complications was comparable, with 17 patients (53.1%) in the drained group and 27 patients (58.7%) in the undrained group; there was no significant difference in the number of patients who had either infectious complications or non-infectious complications. We found this result to be consistent with most of the studies that were reviewed in our systematic search. Most recently, a multicenter European study by Farges *et al*[14] reported that there was no significant difference in the rate of complications between drained and undrained groups of patients undergoing major liver resection. However, the infectious complications were not compared in this study and the risk factors for the overall complications were not analyzed. In our study, there was no significant difference in the number of patients with infectious complications between the two groups, which might be because most of them underwent PTCD (71.9%) as compared to endoscopic techniques (ENBD-15.6% and EBS-0%). EBS in particular has been shown to increase the infectious complication rate as compared to other drainage procedures[18,23,42,43]. Three studies[18-20] from our search reported higher infectious complications in patients who underwent PBD. Four studies[5,18,19,31] reported no significant difference in the non-infectious complication rates between the two groups. In our study, patients undergoing right-sided hepatectomy without PBD had a higher morbidity than patients with PBD, whereas contrary results were obained in the left-sided hepatectomy group. This is consistent with the study carried out by Farges *et al*[14]. Furthermore, while some studies have reported a longer stay in the drained group[20,31], other studies have shown no difference between the two groups[18,19]. In our study, there was no difference in the postoperative hospital stay, reoperation rate and mortality between the two groups.

While PBD was not a risk factor for postoperative complications, preoperative TBIL > 170 μmol/L, a higher Bismuth-Corlette classification and extended liver resection were found to be three independent risk factors for postoperative complications. In our study,

PBD reduced the preoperative serum bilirubin level and other liver function indexes significantly as compared to those on admission. However, this did not translate into a significant reduction in the occurrence of postoperative complications, as compared to the undrained group. Previous studies have shown that preoperative bilirubin levels influence postoperative morbidity and mortality rates[18,34]. However, there is no consensus on the serum bilirubin cut-off level before surgery at which PBD should be undertaken. Some studies recommend undertaking PBD at a bilirubin cut-off of 51.3 μmol/L (3 mg/dL) to minimize complications following major surgery[11,12]. Other studies recommend a bilirubin cut-off of more than 85.5 μmol/L (5 mg/dL)[13]. The serum bilirubin level prior to surgery was 108.1 ± 60.6 μmol/L in our study. Farges *et al*[14] advised that major hepatectomy for jaundiced patients should be delayed until the serum bilirubin level had fallen below 50 μmol/L. Other studies have suggested that PBD should be performed and surgery should be delayed when the preoperative bilirubin level was higher than 171 μmol/L (10 mg/dL)[10,34]. Koyama *et al*[15] advised that adequate recovery of hepatic function depended not only on the duration of obstructive jaundice prior to decompression, but also on the duration of biliary decompression. Some studies have suggested 3-6 wk of preoperative drainage for obstructive jaundice, with even longer periods proposed with a prolonged biliary obstruction before decompression[8,15,16]. In our study, the PBD catheter remained *in situ* for a mean of 15.3 d. In light of the above, it is plausible that postoperative outcomes may have improved further, had we kept the PBD catheter *in situ* longer with a lower preoperative serum bilirubin level. However, we recommend PBD, prior to major hepatectomy, in patients with HCCA with a TBIL above 170 μmol/L.

Gerhards *et al*[5] had reported a higher Bismuth-Corlette classification was associated with postoperative morbidity. Also Li *et al*[32] reported that while PBD alleviated liver injury caused by hyperbilirubinemia, it did not decrease the postoperative morbidity and mortality and concomitant hepatectomy and Bismuth-Corlette classification were independent risk factors linked to surgical risks. This is explainable as a higher Bismuth-Corlette classification warrants a more extensive surgical resection, which resulted in higher morbidity[44]. Indeed, in our study, there were many patients with stage Ⅲ and Ⅳ tumors who underwent extended hepatectomy with caudate lobe resection and vascular resection.

We acknowledge the limitations of our study. First of all, our results derive from a retrospective study and are unavoidably subject to selection bias although a consecutive series was reported. Second, the sample size is relatively small, coming from a single center. Moreover, various factors such as the variable procedures for biliary drainage, treatment of patients at other centers prior to transfer to our center and failure of the initial drainage procedure may have contributed to biases in our study. However, as the baseline characteristics of patients prior to surgery were comparable between the drained and undrained groups, we hope that the effect of these factors on postoperative outcome was minimized. Currently, there continues to be a lack of consensus and recommendations on the use of PBD prior to major liver resection for HCCA. This has been highlighted by our study and review of literature. While an adequately powered randomized controlled trial at a single center may be currently unrealistic, in view of the rarity of this tumor, a multicenter study would go a long way in informing future practice.

In summary, short-term postoperative outcomes after major liver resection for HCCA are not improved by PBD, which is consistent with most of published evidence. Preoperative TBIL > 170 μmol/L and Bismuth-Corlette classification and extended liver resection might be three independent risk factors for postoperative complications. There is a need to undertake multicenter studies to inform future practice.

COMMENTS

Background

Whether preoperative biliary drainage (PBD) should be used in jaundiced patients with hilar cholangiocarcinoma (HCCA) undergoing major liver resection remains unclear.

Research frontiers

To investigate the role of PBD in patients with HCCA undergoing major liver hepatectomy using prospectively maintained database from a specialty center. A retrospective comparative analysis was performed comparing the perioperative and short-term postoperative outcomes of patients with PBD or not.

Innovations and breakthroughs

Based on the study, PBD does not improve short-term postoperative outcomes in patients with HCCA undergoing major liver resection. However, Preoperative total bilirubin (TBIL) > 170 μmol/L, Bismuth-Corlette classification and extended liver resection are three independent risk factors for postoperative complications.

Applications

The advantages of PBD was not found in this study; however, higher preoperative TBIL (> 170 μmol/L) was indeed a risk factor for postoperative complications. In addition, taking into account the nature of a retrospective study, there is a need to undertake well-designed, prospective multicenter studies to inform future practice.

Terminology

PBD is an important method for recovery of liver function in patients with obstructive jaundice, which includes the percutaneous transhepatic cholangiodrainage, endoscopic biliary stenting, endoscopic nasobiliary drainage and surgical drainage.

Peer review

This well-written study investigated the short-time postoperative outcomes and risk factors in jaundiced patients as result of HCCA with PBD or not. It may be of interest for hepatobiliary surgeons worldwide.

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BRIEF ARTICLE

Vascular resection in pancreatic adenocarcinoma with portal or superior mesenteric vein invasion

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Abstract

AIM: To evaluate long-term survival after the Whipple operation with superior mesenteric vein/portal vein resection (SMV/PVR) in relation to resection length.

METHODS: We evaluated 118 patients who underwent the Whipple operation for pancreatic adenocarcinoma at our Department of Hepatobiliary Pancreatic Surgery between 2005 and 2010. Fifty-eight of these patients were diagnosed with microscopic PV/SMV invasion by frozen-section examination and underwent SMV/PVR. In 28 patients, the length of SMV/PVR was ≤ 3 cm. In the other 30 patients, the length of SMV/PVR was > 3 cm. Clinical and survival data were analyzed.

RESULTS: SMV/PVR was performed successfully in 58 patients. There was a significant difference between the two groups (SMV/PVR ≤ 3 cm and SMV/PVR > 3 cm) in terms of the mean survival time (18 mo *vs* 11 mo) and the overall 1- and 3-year survival rates (67.9% and 14.3% *vs* 41.3% and 5.7%, $P < 0.02$). However, there was no significant difference in age (64 years *vs* 58 years, $P = 0.06$), operative time (435 min *vs* 477 min, $P = 0.063$), blood loss (300 mL *vs* 383 mL, $P = 0.071$) and transfusion volume (85.7 mL *vs* 166.7 mL, $P = 0.084$) between the two groups.

CONCLUSION: Patients who underwent the Whipple operation with SMV/PVR ≤ 3 cm had better long-term survival than those with > 3 cm resection.

Key words: Pancreatic adenocarcinoma; Whipple operation; Vascular resection

Core tip: Pancreatic adenocarcinoma can infiltrate the portal vein (PV) or superior mesenteric vein (SMV). In order to achieve negative surgical margins, the Whipple operation combined with SMV/PV resection (SMV/PVR) is usually performed. The long-term survival rate of patients with SMV/PV involvement in relation to the length of resection remains controversial.

Pan G, Xie KL, Wu H. Vascular resection in pancreatic adenocarcinoma with portal or superior mesenteric vein invasion. *World J Gastroenterol* 2013; 19(46): 8740-8744 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i46/8740.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i46.8740

INTRODUCTION

Pancreatic adenocarcinoma is a malignant neoplasm that is one of the most common causes of cancer-related death. Unfortunately, there are no symptoms in the early period of the disease, so fewer patients have the chance of achieving negative margin resection. The reason for the lower treatment rate is that many patients have liver metastases, lymph node involvement, invasion of retroperitoneal tissue, and portal vein (PV)/superior mesenteric vein (SMV) invasion when they are diagnosed[1-4]. Since the Whipple operation combined with SMV/PV resection (SMV/PVR) and reconstruction for pancreatic adenocarcinoma was first reported in 1951[5], the value

of SMV/PVR has remained controversial[6,7]. In the past, tumor invasion of the PV/SMV was considered a contraindication to tumor resection because of the high rate of recurrence and poor prognosis. Recently, some departments have argued that combination of the Whipple operation with SMV/PVR can achieve similar long-term survival to the Whipple operation alone without any increase in morbidity and mortality[8-10].

However, the suitable length of SMV/PVR is under discussion. In this study, we evaluated the outcome in patients who underwent the Whipple operation with SMV/PVR ≤ 3 cm compared with > 3 cm. We aimed to clarify long-term survival of patients with SMV/PV invasion in relation to the depth of venous involvement.

MATERIALS AND METHODS

Patients and methods

From January 2005 to December 2010, 118 consecutive patients who underwent the Whipple operation for pancreatic adenocarcinoma were analyzed at the Department of Hepatobiliary Pancreatic Surgery, Sichuan University. There were 70 men and 48 women with a median age of 53 years (range, 23-78 years). According to preoperative image evaluation and intraoperative frozen-section examination, 60 patients with pancreatic adenocarcinoma underwent the Whipple operation alone. Twenty-eight patients underwent the Whipple operation combined with SMV/PVR ≤ 3 cm. Thirty patients underwent the Whipple operation with SMV/PVR > 3 cm.

Preoperative evaluation

Preoperative evaluation included a careful physical examination; a series of blood tests such as tumor markers (carcinoembryonic antigen and carbohydrate antigen 19-9), liver function, and thrombin; and chest radiography, abdominal ultrasonography, contrast computed tomography, and electrocardiography. Sometimes magnetic resonance cholangiopancreatography or endoscopic retrograde cholangiopancreatography was selectively performed.

Indications and operative technique

The exclusion criteria were as follows: (1) extrapancreatic disease such as liver and peritoneal metastases; (2) Whipple operation with SMV/PVR tangential resection; and (3) Whipple operation combined with adjuvant chemotherapy or chemoradiotherapy. We also excluded patients with a previous unsuccessful attempt at pancreatectomy because they could be exposed to different early morbidity or distant prognosis.

The Whipple operation was performed in all of the consecutive patients. Hemigastrectomy was performed, and the bile duct was divided above the cystic duct. An end-to-side pancreaticojejunostomy, end-to-side hepaticojejunostomy, and side-to-side gastrojejunostomy were performed as classic reconstruction after the Whipple operation[10]. Vascular consecutiveness was recovered by a direct end-to-end anastomosis. None of the patients in our group used low-molecular-weight heparin after venous reconstruction.

Table 1 Comparison of characteristics between the two groups

Demographics	SMV/PVR ≤ 3 cm (*n* = 28)	SMV/PVR > 3 cm (*n* = 30)	*P* value
Sex (M/F)	21/7	20/10	0.082
Age (yr)	64 (range 31-78)	58 (range 38-77)	0.063
Tumor size (cm)	3.1 (range 2-6)	3.7 (range 3-7)	0.051
Tumor stage			0.056
Ⅰ	5	2	
Ⅱ	16	6	
Ⅲ	7	22	
Ⅳ	0	0	
Curability			0.067
R0	26	22	
R1+	2	8	
Depth of venous involvement			0.032
Tunica adventitia	4	2	
Tunica media	14	12	
Tunica intima	10	16	
Lymph node invasion	25%	73%	0.043
Operative time (min)	435	477	0.064
Blood loss (mL)	300	383	0.071
Transfusion (mL)	85.7	166.7	0.084

SMV/PVR: Superior mesenteric vein/portal vein resection.

Statistical analysis

Perioperative data such as pathological data, length of hospital stay, operative blood loss, volume of blood transfusion, morbidity, and mortality were obtained from medical records. The long-term survival outcomes were obtained through postoperative follow-up at outpatient clinics or on the telephone. The outcomes in the two groups were analyzed using the χ^2 test. All statistical analyses were performed using SPSS version 19.0, when $P < 0.05$ was considered statistically significant.

RESULTS

The demographic and operative characteristics of the patients who underwent SMV/PVR ≤ 3 cm and > 3 cm are shown in Table 1. The median age of the two groups was 64 years (range, 31-78 years) and 58 years (range, 38-77 years), respectively. The median size of the pancreatic tumors was 3.1 cm (range, 2-6 cm) and 3.7 cm (range, 3-7 cm), respectively. The median length of venous resection was 2.5 cm (range, 1-3 cm) and 3.8 cm (range, 3.5-5 cm), respectively. The mean operation time for patients with SMV/PVR ≤ 3 cm and > 3 cm was 435 and 477 min, respectively. The mean blood loss was 300 and 383 mL, respectively. There was no significant difference in operative time, blood loss, transfusion volume, and tumor stage between the two groups. However, there were significant differences in lymph node invasion, depth of venous involvement, and length of SMV/PVR between the two groups. By multivariate analysis, the length of venous resection was the most important prognostic factor.

The postoperative complication and mortality rates

Table 2 Surgical mortality and morbidity in 58 patients who underwent standard Whipple operation with portal vein resection

Morbidity	SMV/PVR ≤ 3 cm (n = 28)	SMV/ PVR > 3 cm (n = 30)
Hemorrhage	1	2
Hypertension with upper gastrointestinal bleeding	0	1
Pancreatic fistula	2	3
Wound infection	1	1
Reoperation	1	1
Recurrence	21	27
Median hospital stay (d)	18 (range 8-32)	18 (range 11-43)

SMV/PVR: Superior mesenteric vein/portal vein resection.

are shown in Table 2. Hemorrhage from the surgical site occurred in three patients after the operation, and one of these died 7 d after surgery. Two patients underwent reoperation. In the patients with SMV/PVR ≤ 3 cm, hypertension with upper gastrointestinal bleeding occurred in one patient who underwent spleen vein transection without vascular remodeling.

The overall 1- and 3-year survival rates for patients who underwent the standard Whipple operation combined with SMV/PVR ≤ 3 cm (n = 28) and SMV/PVR > 3 cm (n = 30) were 67.9% and 14.3%, and 41.3% and 5.7%, respectively. The mean survival of patients with SMV/PVR ≤ 3 cm and SMV/PVR > 3 cm was 18 and 11 mo, respectively. There was a significant difference in survival between the two groups (Figure 1; P = 0.02).

DISCUSSION

Pancreatic adenocarcinoma is a malignant disease and negative resection margin is still the best treatment option at present. In the past, only 10%-20% of patients with pancreatic adenocarcinoma could undergo surgery because of distant metastases and vascular involvement[11-13]. Due to the intimate relationship of the pancreatic head and uncinate, the PV is always infiltrated[9]. Surgeons previously considered that pancreatic adenocarcinoma with venous involvement was a contraindication to surgery. They also considered that venous invasion always hindered complete tumor removal. Recent improvements in preoperative imaging and surgical techniques have resulted in the standard Whipple operation with SMV/PVR offering the possibility of achieving negative margin resection in patients with pancreatic adenocarcinoma and SMV or PV involvement, without a relevant increase in morbidity and mortality[14-16]. Our study also supports this. However, the suitable length of SMV/PVR is under discussion.

The main conclusion of our retrospective analysis was that patients who underwent the standard Whipple operation with SMV/PVR (Group 1) had similar survival rates and negative resection margins when compared with patients without PV involvement (Group 2). In our study, the median survival time in Group 1 (n = 58) and Group 2 (n = 60) was 19 and 21 mo, respectively. In addition, the 1- and 3-year survival rates in the two groups were 63.3%

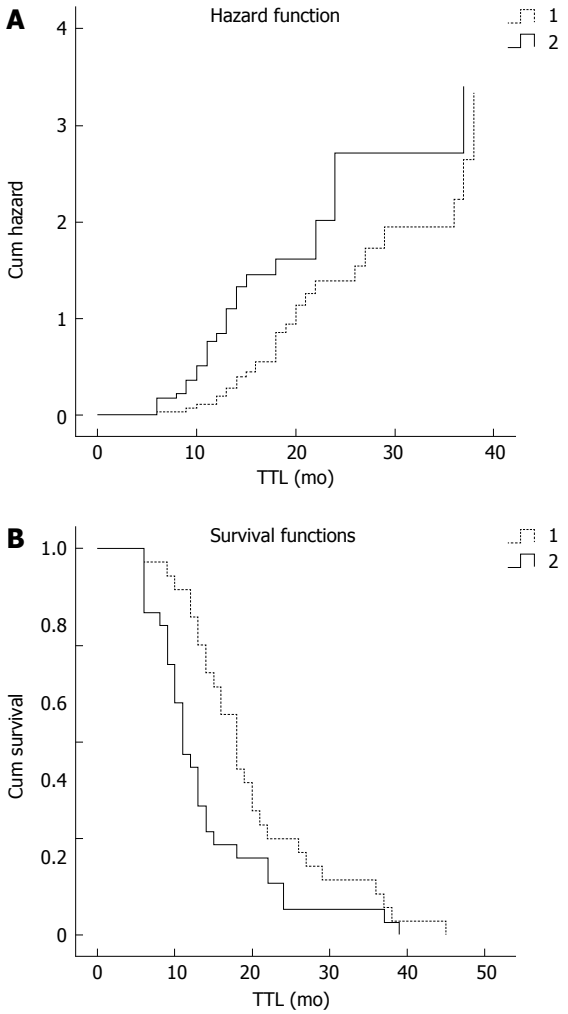

Figure 1 Survival of patients with portal vein resection. A: Patients with superior mesenteric vein/portal vein resection (SMV/PVR) ≤ 3 cm (Dotted line, n = 28) had more risk factors compared with patients with > 3 cm resection (solid line, n = 30); B: SMV/PVR ≤ 3 cm (Dotted line, n = 28) was significantly better than > 3 cm resection (solid line, n = 30).

and 14.3%, and 69.3% and 18.4%, respectively. There were no significant differences in survival between the two groups (P > 0.05). At the same time, the blood loss, volume of transfusion, and surgical mortality and morbidity did not increase obviously in Group 1. However, venous resection combined with reconstruction (Group 1) cost more compared with Group 2, but there was no significant difference in survival between the two groups (P > 0.05). Therefore, we considered that patients with SMV/PVR had similar long-term survival to those without SMV/PVR.

Another conclusion is that patients who underwent the Whipple operation with SMV/PVR ≤ 3 cm (Group 3) achieved better long-term survival than those with SMV/PVR > 3 cm (Group 4). Median survival time in Group 3 (n = 28) and Group 4 (n = 30) was 18 and 11 mo, respectively. There was a significant difference in survival between the two groups (P < 0.02). Meanwhile, the patients with SMV/PVR ≤ 3 cm had more risk factors compared with those with SMV/PVR > 3 cm (P < 0.05)

(Figure 1). For example, the ratio of lymph node invasion between the two groups was 25% and 73%, respectively, and this difference was significant ($P < 0.05$). Therefore, we consider that 3 cm is a suitable length of SMV/PVR.

Illuminati *et al*[17] reported that standard Whipple operation combined with PV or SMV resection can be performed when venous involvement does not exceed 2 cm. They have suggested that more complex vein reconstruction could lead to a greater rate of postoperative complications. They have also clarified that 2 cm is the maximal extent that allows one to achieve margin-free resection with simple vascular reconstruction and tension-free anastomosis. However, in our study, we achieved tension-free end-to-end anastomosis by dissociating the root of the SMV when the length of PVR was about 5 cm. Recently, some institutions have suggested that a distance of up to 8 cm can achieve primary anastomosis by this procedure[6,18]. Thus, we believe that the main factor influencing the length of venous resection is not the surgical technique itself but the long-term survival. Our study indicated that patients who underwent the Whipple operation with SMV/PVR ≤ 3 cm achieved better long-term survival.

Some studies have reported the use of venous interposition graft[10,19-21]. They have proposed that SMV/PVR > 5 cm and venous collateral formations should use venous interposition grafting[14]. The autogenous venous graft often uses the internal jugular and the greater saphenous veins. The prosthetic venous reconstruction material is usually polytetrafluoroethylene. However, Riediger *et al*[6] considered that the Whipple operation has a risk of abdominal infection, and the use of venous prostheses might increase this complication. In our series, all the patients with PVR had a primary end-to-end reconstruction without any autogenous graft or venous prosthesis.

Shibata *et al*[10] divided SMV/PVR into four types: (1) above and below the level of the splenic vein; (2) above the level of the splenic vein; (3) below the level of the splenic vein; and (4) tangential resection. In our present series, among the 58 patients who underwent the Whipple operation and venous resection, 10 underwent PVR above and below the level of the splenic vein. Among these 10 patients, 4 patients underwent vena lienalis ligatured and transected without splenic vein reconstruction, and six patients had vena lienalis ligation and resection with splenic artery wedge resection in order to reduce the blood flow to the spleen. In the four patients without splenic vein reconstruction, one had local hypertension and upper gastrointestinal bleeding and died in the 14 d after the operation. It has also been reported that division of the splenic vein without splenectomy might lead to portal thrombosis[22-24], but this did not occur in our study. Our experience suggests that splenic vein ligatured and transected with splenic artery reconstruction should be performed when the confluence of spleen vein is involved.

Shibata *et al*[10] have proposed that the degree of venous involvement can be divided into three types: no mural invasion, intramural invasion without tunica intima involvement, and transmural invasion. Several documents have reported that patients with tunica intima infiltration could not obtain good long-term survival[10,14]. In our study, no patient survived beyond 8 mo, when the tunica intima was involved.

Bao *et al*[23] have suggested that mesenteric artery involvement > 90°, as visualized by computed tomography, implies that we cannot achieve disease-free resection. Today, most surgeons agree that tumor invasion of the mesenteric artery is a contraindication to the Whipple operation[17,25,26]. It is considered that the mesenteric artery is often encircled by a neural plexus and lymph nodes. Therefore, artery involvement is always combined with neural plexus and lymph node invasion, and it is difficult to achieve a negative resection margin. It has also been reported that patients with positive lymph nodes have worse overall survival than patients without lymph node invasion, and extensive lymphadenectomy and nerve plexus resection might lead to serious diarrhea and poorer quality of life. Therefore, other treatments such as neoadjuvant and adjuvant chemotherapy could be used in patients with arterial invasion.

In conclusion, we showed that patients with pancreatic adenocarcinoma and venous invasion who underwent the standard Whipple operation with SMV/PVR had similar long-term survival than patients without venous involvement. In addition, patients who underwent the Whipple operation with SMV/PVR ≤ 3 cm achieved better long-term survival than those with > 3 cm resection.

COMMENTS

Background

Pancreatic adenocarcinoma is a malignant neoplasm. Due to the close relationship between the pancreas and the superior mesenteric vein (SMV) and portal vein (PV), pancreatic cancer can infiltrate the PV/SMV.

Research frontiers

The Whipple operation and SMV/PV resection (SMV/PVR) has been considered the standard operation for patients with pancreatic adenocarcinoma and PV or SMV involvement. However, the long-term survival rate of patients with PV/SMV involvement in relation to the length of SMV/PVR is under discussion.

Innovations and breakthroughs

The authors studied 118 patients who underwent the Whipple operation for pancreatic adenocarcinoma between 2005 and 2010. Fifty-eight patients were diagnosed with microscopic SMV/PV invasion by frozen-section examination and underwent SMV/PVR. Twenty-eight of these 58 patients underwent SMV/PVR < 3 cm. Thirty patients underwent SMV/PVR > 3 cm. The authors performed this retrospective study to clarify the long-term survival rate of patients with SMV/PV involvement in relation to the length of SMV/PVR by analyzing the clinical and survival data of those 58 patients.

Applications

Patients who underwent the Whipple operation with SMV/PVR ≤ 3 cm achieved better long-term survival than those with SMV/PVR > 3 cm.

Peer review

The overall contents are interesting with clinical significance. The data from Whipple procedure only group should be included in tables to compare with SM-PVR groups, in particular for the parameters of time to progress or recurrence and overall survival time. In particular, the comparison of overall survival time between Whipple only and Whipple plus SM-PVR groups.

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BRIEF ARTICLE

Psychometric hepatic encephalopathy score for diagnosis of minimal hepatic encephalopathy in China

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Abstract

AIM: To construct normal values for the tests of the psychometric hepatic encephalopathy score (PHES) and to evaluate its usefulness in the diagnosis of minimal hepatic encephalopathy (MHE) among Chinese individuals with cirrhosis.

METHODS: The five tests of PHES, number connection test-A (NCT-A), number connection test-B, serial dotting test, line tracing test and digit symbol test (DST), were administered to all enrolled subjects in a quiet room with sufficient light. Cirrhotic subjects with overt HE were excluded by the West-Haven criteria and a detailed neurological examination. Based on the nomograms of healthy volunteers, the patients were classified as having MHE when their PHES was less than -4.

RESULTS: In total, 146 healthy volunteers completed all the PHES tests. Age and education years were confirmed to be predictors of all five tests. In total, 53 patients with liver cirrhosis completed the PHES. Of the patients with liver cirrhosis, 24 (45.3%), 22(41.5%) and 7(13.2%) had Child-Pugh grades A, B and C, respectively. MHE was diagnosed in 26 patients (49.1%). Compared with compensated cirrhotic patients (Child A), decompensated cirrhotic patients (Child B and C) had a higher proportion of MHE (65.5% *vs* 29.2%). No differences in age and education years were found between the MHE and non-MHE groups. NCT-A and DST were able to diagnose MHE with a sensitivity of 76.9% and a specificity of 96.3% (AUC = 0.866, K = 0.735).

CONCLUSION: The proportion of MHE is associated with liver function. NCT-A and DST are simple tools that can be used for the diagnosis of MHE in China.

Key words: Cirrhosis; Minimal hepatic encephalopathy; Neuropsychological tests; Psychometric hepatic encephalopathy score; Number connection test; Digit symbol test

Core tip: The psychometric hepatic encephalopathy score (PHES) has been standardized in several countries, but requires further validation in China. The authors aimed to evaluate the usefulness of PHES for the diagnosis of minimal hepatic encephalopathy (MHE) among Chinese patients with liver cirrhosis. In China, the results of the five neuropsychological tests of PHES were influenced by age and educational status. In total, 49.1% of the patients with cirrhosis were classified as

having MHE, and the proportion of MHE was associated with the severity of liver function. Number connection test-A and digit symbol test are simple and useful tools that can be used for the diagnosis of MHE in China.

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INTRODUCTION

Minimal hepatic encephalopathy (MHE) is a highly prevalent asymptomatic disturbance in patients with liver cirrhosis. MHE is associated with impaired health-related quality of life and driving capability and can predict the development of overt hepatic encephalopathy (OHE)[1-4]. MHE is not detectable by routine physical or neurological examinations, and a specific neuropsychological/neurophysiological test is needed for its diagnosis[5-7]. The psychometric hepatic encephalopathy score (PHES) is internationally recommended as the gold standard for the diagnosis of MHE[8,9].

The PHES is composed of five tests, number connection test-A (NCT-A), number connection test-B (NCT-B), serial dotting test (SDT), line tracing test (LTT) and digit symbol test (DST). PHES can be used to assess motor speed, motor accuracy, concentration, attention, visual perception, visual-spatial orientation, visual construction and memory[10], which are related to most of neuropsychological impairments in MHE. The PHES has been standardized in several countries, such as Germany, Italy, Spain, India, Korea and Mexico. However, in China further validation is needed. The aims of this study were to construct and validate a dataset of normal values for the PHES in a healthy Chinese population and to evaluate the usefulness of PHES for the diagnosis of MHE among Chinese patients with liver cirrhosis.

MATERIALS AND METHODS

Subjects

Healthy volunteers: The healthy volunteers that were recruited for the control group included people who visited the Health Promotion Center at the First Affiliated Hospital of Anhui Medical University in Hefei, China, for routine health examinations, and through word-of-mouth referrals. The following exclusion criteria were applied for the control group: (1) Presence of chronic liver diseases, neurological or psychiatric diseases, or other diseases that can affect cognitive function; (2) A past history of chronic liver disease, neurologic or psychiatric disorders; (3) Consumption of psychotropic drugs; (4) Alcohol consumption > 50 g/d within the past 3 mo; and (5) Inability to read and write.

Liver cirrhosis group: Consecutive inpatients from the Department of Gastroenterology and Hepatology were recruited. Patients with OHE, which was defined according to the West-Haven criteria[11], were excluded. The diagnosis of liver cirrhosis was based on a combination of physical examination, laboratory tests, medical imaging and endoscopic evidence or on liver histology, if available. The following exclusion criteria were applied for the liver cirrhosis group: (1) A history of OHE, upper gastrointestinal hemorrhage or spontaneous bacterial peritonitis during the past 2 wk; (2) Consumption of lactulose, psychoactive drugs or any antibiotics during the past 2 wk; (3) Presence of neurological or psychiatric diseases, such as Alzheimer's disease, Parkinson's disease and nonhepatic metabolic encephalopathy, or a mini-mental status examination (MMSE) score < 25 points; (4) Presence of significant comorbidity, such as heart, respiratory, or renal failure; (5) Presence of hepatocellular carcinoma or other malignancy, previous TIPS or shunt surgery; (6) Alcohol consumption > 50 g/d within the past 3 mo; and (7) Inability to read and write.

All the subjects, both healthy volunteers and patients with liver cirrhosis, were required to have a fair knowledge of numbers and the Chinese alphabet. The research protocol was approved by the ethics committee of the hospital in accordance with the ethical guidelines of the Declaration of Helsinki. Written informed consent to participate was obtained from each subject.

Neuropsychological tests

All the five tests of PHES were administered to all the enrolled subjects in the same sequence. The tests were conducted on a one-to-one basis in a quiet room with sufficient light. A specially trained medical doctor assisted the enrolled subjects in finishing these tests.

As some of our enrolled subjects were not familiar with the English alphabet, we replaced the alphabet in NCT-B with the Chinese alphabet in the same order[12]. The results of the NCT-A, NCT-B, and SDT were measured as seconds, including the time needed to correct any errors, and the result of DST was measured as points. The results of the LTT were measured as both the time needed to complete the test (LTTt, seconds) and as the error score (LTTe), LTT = (1 + LTTe/100) × LTTt[13]. Accordingly, a higher result of DST equals better performance, and lower results on the other tests equal better performance. Formulas were constructed to predict the expected results of the five neuropsychological tests. These values were then used as references to which the results from the patients with liver cirrhosis were compared.

The result of DST within ± 1SD from the mean of the control performance was scored as 0 points. Results between -1 and -2SD, between -2 and -3SD and worse than -3SD were scored as -1, -2 and -3, respectively. A result better than mean + 1SD was scored as +1.

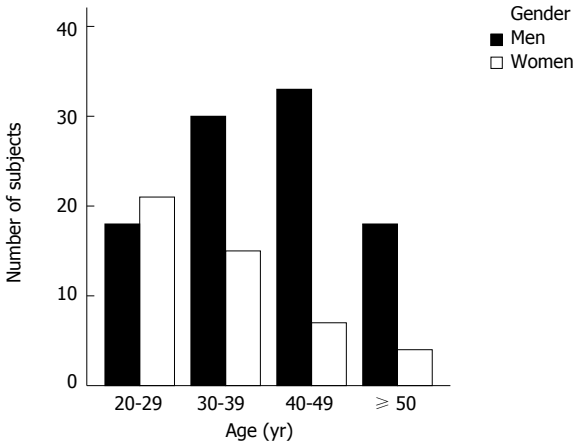

Figure 1 Distribution of volunteers according to age.

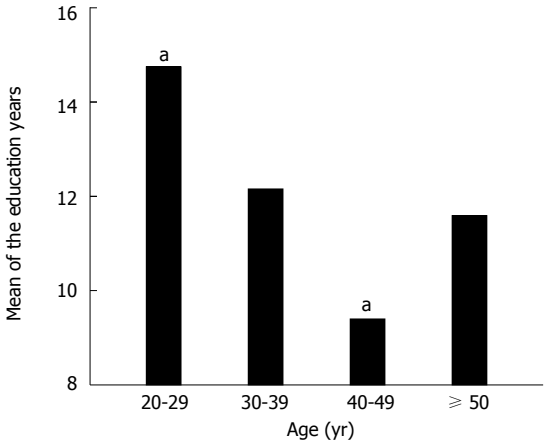

Figure 2 Comparison of education years between healthy volunteers of various age groups ([a]*P* < 0.05).

The results (NCT-A, NCT-B, SDT and LTT) within ± 1SD from the mean of the control performance were scored as 0 points. Results between +1 and +2SD, between +2 and +3SD, and worse than +3SD were scored as -1, -2 and -3 points, respectively. Those better than mean -1SD were scored as +1 point[10]. The final score of PHES was generated from the sum of the scores of five tests, which ranged between +5 and -15.

Blood tests and biochemical examinations

On the day of neuropsychological testing, venous blood was taken for routine liver function tests, hematologic parameters and venous ammonia concentration. Venous ammonia was measured within 30 min after blood sampling.

Statistical analysis

Statistical analyses were performed using the statistical package for the social science (SPSS version 11.0; SPSS, Chicago, IL, United States). Data are expressed as mean ± SD or as proportion. ROC analysis was performed with results of NCT-A, NCT-B and DST comparing to PHES. Continuous and categorical variables were compared using the *t* test, the one-way ANOVA test, the Mann-Whitney *U*-test and the χ^2-test, respectively. Levene's test was used in the evaluation of differences in variance. Non-parametric tests were applied if homogeneity of variance assumptions were not met. Multiple liner regression models were used to predict the value of each test for patients with liver cirrhosis. The difference between the expected and observed results for each test was divided by the corresponding SD of the healthy reference population. Kappa statistics were used to study the agreement between the PHES and NCT-A, NCT-B, DST. A two-sided *P* value < 0.05 was considered significant.

Table 1 Correlations between psychometric tests and age and education years

	NCT-A	NCT-B	LTT	SDT	DST
Age	0.510	0.478	0.336	0.322	-0.647
Education	-0.409	-0.355	-0.358	-0.374	0.585

The data are presented as Pearson's correlation coefficients; $P < 0.05$; NCT-A: Number connection test-A; NCT-B: Number connection test-B; SDT: Serial dotting test; LTT: Line tracing test; DST: Digit symbol test.

RESULTS

PHES of healthy volunteers and the relationships between PHES and age and education

Of 154 healthy volunteers who were recruited, 8 were not able to complete NCT-B and as such, only the remaining 146 volunteers were included. The age and education years of the 146 volunteers were 37.3 ± 10.5 (range 20-67) and 12.0 ± 4.0 (range 2-19) years, respectively, and 99 were men (67.8%). The distribution of subjects according to age was as follows: 20-29 years, 39 (26.7%); 30-39 years, 45 (30.8%); 40-49 years, 40 (27.4%); and ≥ 50 years, 22 (15.1%) (Figure 1). The education years according to age are presented in Figure 2.

The results of NCT-A, NCT-B, LTT, SDT and DST were 38.289 ± 13.694, 55.846 ± 17.798, 33.287 ± 8.286, 38.035 ± 5.774 and 55.0 ± 14.3, respectively. The results of the five tests were significantly correlated with age and education, and the Pearson's correlation coefficients are shown in Table 1. In all age categories, the results of all five tests were not significantly correlated with gender ($P > 0.05$). The variables that affected the results of a neuropsychological test were included in the multiple liner regression models, and the final formulas are shown in Table 2. As shown in Table 2, age and education years were predictors of the results of the five neuropsychological tests in healthy volunteers. As shown in Figure 3, younger age and better education were associated with better DST results.

In the healthy volunteer group, the score of PHES was not correlated with education years ($P = 0.992$) or age ($P = 0.595$). Additionally, the PHES did not differ between men and women ($P = 0.589$).

Table 2 Equations for predicting test results from age and education years

Test	Equation	SD
NCT-A	27.861 + 0.548 × age - 0.821 × education	7.581
NCT-B	42.816 + 0.672 × age - 0.971 × education	9.173
SDT	38.937 + 0.113 × age - 0.423 × education	2.408
LTT	33.242 + 0.182 × age - 0.559 × education	3.455
DST	63.020 - 0.672 × age + 1.421 × education	10.608

NCT-A: Number connection test-A; NCT-B: Number connection test-B; SDT: Serial dotting test; LTT: Line tracing test; DST: Digit symbol test; Age and education are expressed in years.

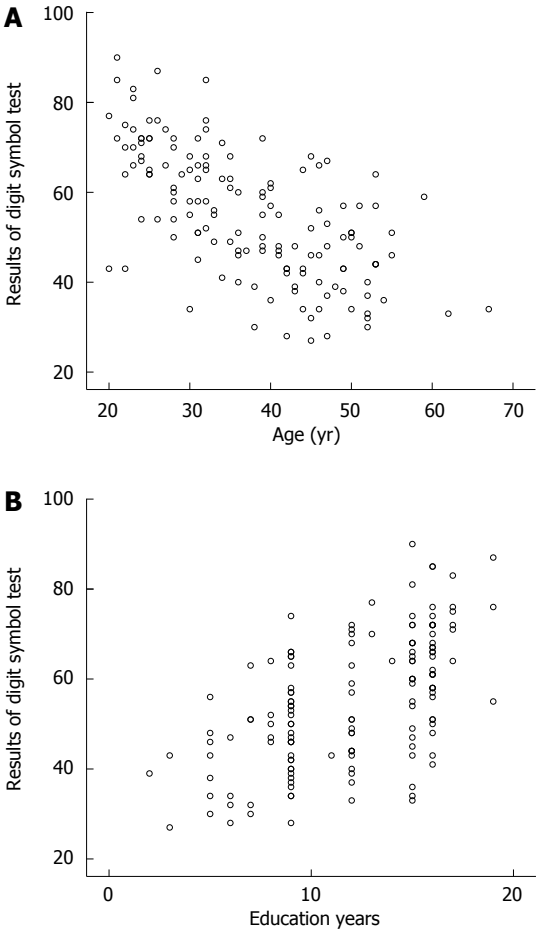

Figure 3 Distribution of the results from the digit symbol test in healthy volunteers according to age (A) and education years (B).

Factors associated with MHE

Of 56 inpatients with liver cirrhosis that were enrolled, 3 were not able to complete NCT-B and thus were not considered further. All tests of the PHES were completed by 53 patients with cirrhosis whose age and education years were 45.6 ± 8.2 years (range 27-62) and 8.2 ± 3.6 years (range 0-15), respectively. The study group comprised 50 (94.3%) men.

The score of PHES in the healthy volunteer group was -0.6 ± 3.7 (median, 0; range -11 to +5). The score of PHES in the liver cirrhosis group was -5.6 ± 4.9 (median, -4; range -13 to +4), significantly lower than that in the volunteer group (Mann-Whitney $U = 1476.00$, $P = 0.000$). In the healthy volunteer group, the lower boundary of the 95% range between mean - 2SD and mean + 2SD was -4.0. Using a cutoff for MHE of < -4, 26 of the 53 patients with liver cirrhosis were diagnosed with MHE (49.1%).

Table 3 Clinical characteristics of patients with liver cirrhosis

	MHE	Non-MHE		*P* value
Age (yr)	45.3 ± 8.0	45.9 ± 8.5	$t = 0.289$	0.774
Education (yr)	8.3 ± 4.4	8.4 ± 2.8	Mann-Whitney $U = 348.000$	0.956
Ammonia (μmol/L)	74.2 ± 64.2	52.9 ± 24.2	$t = 1.086$	0.288
Child-Pugh grade			$\chi^2 = 6.943$	0.008
Child A	7	17		
Child B/C	19	10		
Esophageal varices				0.584[1]
With esophageal varices	15	19		
Without esophageal varices	2	1		
HBV			$\chi^2 = 0.048$	0.827
HBV positive	19	19		
HBV negative	7	8		
Antiviral therapy				> 0.05[1]
With antiviral therapy	4	4		
Without antiviral therapy	15	15		

[1]By Fisher's exact test; MHE: Minimal hepatic encephalopathy; HBV: Hepatitis B virus.

The proportion of patients with MHE increased with the increase in the Child-Pugh grade. Specifically, 7 had Child-Pugh grade A (7/24, 29.2%), 14 had Child-Pugh grade B (14/22, 63.6%) and 5 had Child-Pugh grade C (5/7, 71.4%). Compared with compensated cirrhotic patients (Child A), decompensated cirrhotic patients (Child B and C) had a higher proportion of MHE (19/29 *vs* 7/24; $\chi^2 = 6.943$, $P = 0.008$). No differences in age and education years were found between the MHE and non-MHE groups ($P > 0.05$). Venous ammonia concentration was measured in 26 cirrhotic patients and was found to be similar between the MHE and non-MHE groups ($t = 1.086$, $P = 0.288$). In 37 patients with cirrhosis who underwent endoscopic examination, the prevalence of MHE was not associated with esophageal varices ($P = 0.584$ by Fisher's exact test). In total, 38 of 53 cirrhotic patients were hepatitis B virus (HBV) positive, while 15 were HBV negative. The prevalence of MHE was similar between the HBV positive and negative groups ($\chi^2 = 0.048$, $P = 0.827$) and the prevalence of MHE was not influenced by antiviral therapy. Table 3 shows the characteristics of patients with or without MHE.

Comparisons of PHES with NCT-A, NCT-B and DST

International consensus recommends that at least two of the NCT-A, NCT-B, DST and block-design test (BDT) should be used for the diagnosis of MHE[8]. Because the BDT is not easy to use, we compared PHES assessment

Table 4 Comparisons between psychometric hepatic encephalopathy score and number connection test-A, number connection test-B, and digit symbol test

		Sensitivity	Specificity	AUC	*K* value
Both of the two tests were abnormal	NCT-A + NCT-B	0.538	0.963	0.751	0.505
	NCT-A + DST	0.077	1.000	0.538	0.078
	NCT-B + DST	0.154	1.000	0.577	0.156
All of the three tests were abnormal	NCT-A + NCT-B+DST	0.077	1.000	0.538	0.078
At least one of the two tests was abnormal	NCT-A/NCT-B	0.923	0.741	0.832	0.661
	NCT-A/DST	0.769	0.963	0.866	0.735
	NCT-B/DST	0.769	0.741	0.755	0.510
At least one of the three tests was abnormal	NCT-A/NCT-B/DST	0.923	0.741	0.832	0.661
At least two of the three tests were abnormal	NCT-A/NCT-B/DST	0.615	0.963	0.789	0.582

PHES: Psychometric hepatic encephalopathy score; NCT-A: Number connection test-A; NCT-B: Number connection test-B; DST: Digit symbol test; AUC: Area under the curve.

using NCT-A, NCT-B and DST. Based on the normal range of healthy volunteers, the result of a single test was classified to be abnormal if the score was less than -1 point[10]. Using the NCT-A and DST, we were able to diagnose MHE with a sensitivity of 76.9% and a specificity of 96.3% (AUC = 0.866, K = 0.735), if at least one of the two tests was abnormal (Table 4).

DISCUSSION

MHE refers to the cognitive defects in patients with cirrhosis and/or portal-systematic shunting that can be diagnosed after the exclusion of OHE and alternative diagnoses for neuropsychological impairment[8,11]. Despite the impact of MHE, most cirrhotic patients are not routinely tested for MHE and remain untreated, because of the lack of standardization of normal values, simple tools and expertise to administer tests[14]. Validations of reference norms for neuropsychological tests may increase the likelihood for detection of MHE. The PHES is a neuropsychological test that was specifically designed and recommended for diagnosis of MHE[8,15]. The PHES has been validated in Germany, Italy, Spain and other countries. To date, the number of studies focused on the prevalence of MHE in Chinese patients with liver cirrhosis is limited, and the validation of PHES in China is needed. Due to the high prevalence of liver cirrhosis and the impact of MHE, it is important to screen for MHE in China. As such, we sought to construct a normative dataset for PHES in healthy Chinese volunteers and to evaluate the value of PHES in the diagnosis of MHE among Chinese patients with liver cirrhosis. In our study, we found that age and education years were predictors of all five tests included in PHES. However, no differences in age and education years were found between the MHE and non-MHE groups. The proportion of patients with MHE was associated with the severity of liver function.

Age and educational status are widely recognized to be associated with the results of neuropsychological tests and accordingly age- and -education -matched normal values of healthy controls are recommended[8]. In the study from Spain, the results of NCT-A and NCT-B were better in males than in females. In our present study, all five neuropsychological tests of the PHES were influenced by age and education. However, they did not differ between males and females in all age categories. As such, age and education, which affected the results of the neuropsychological tests, were included in the multiple linear regression model and formulas used to establish the expected values. In the healthy volunteer group, the PHES was not affected by age, education and gender. In this study, normative data that were matched for age and education years were used, and no differences were found between patients with and without MHE. Therefore, we conclude that in the Chinese population, age and education influence the neuropsychological tests included in the PHES, but are not associated with the score of PHES and the presence or absence of MHE.

When the cutoff was set at -4, PHES had good sensitivity and specificity for diagnosing MHE[10]. This is the same cutoff that was used by the majority of studies focusing on the use of PHES for screening of MHE[10,12,13,15-21]. In this study, the lower boundary of the 95% range between mean-2SD and mean+2SD in the volunteer group was -4.0. Accordingly, patients with liver cirrhosis were diagnosed with MHE on the basis of PHES scores lower than -4. MHE was diagnosed in 49.1% of patients with liver cirrhosis. This is similar to a study from India, in which 48% of cirrhotic patients were diagnosed with MHE[20]. However, a lower incidence of MHE (25.6%) was reported in a study from Korea[12]. One reason might be that the liver function of patients in the studies was different. While 80.6% had Child A in the Korean study, the proportion of Child A in our study and the Indian study were 45.3% and 22.0%, respectively. This higher proportion of Child A may account for the low incidence of MHE diagnosed.

In our study, the proportion of patients with MHE increased with the increase in the Child-Pugh grade as follows: 7 of 24 patients (29.2%) with compensated liver cirrhosis (Child-Pugh grade A) and 19 of 29 patients (65.5%) with decompensated liver cirrhosis (Child-Pugh grades B and C) (P = 0.008). This finding is consistent with those of previous studies[17,22]. MHE was further confirmed to be affected by liver function. The pathogenesis of HE is multifactorial, and ammonia is considered

an important risk factor[23]. However, the relationship between blood ammonia concentration and MHE is still controversial[12,24-26]. Ammonia reaches the systemic circulation and accumulates in the central nervous system via esophageal varices[27]. In the present study, we found that MHE did not correlate with the presence of esophageal varices and venous ammonia levels. In MHE patients, the blood brain barrier may be breached[23], enabling ammonia to diffuse across the blood-brain barrier into the brain more freely[28]. As such, the venous ammonia concentration of patients with MHE may be similar to patients without MHE.

International consensus meetings have recommended the use of the PHES for diagnosing MHE[8,9]. The Vienna consensus has also recommended that at least two of four tests (NCT-A, NCT-B, DST and BDT) should be used for the diagnosis of MHE[8]. Three of the four tests, NCT-A, NCT-B and DST, have been commonly used for the detection of MHE. The result of a single test was regarded to be abnormal if the result was beyond the 2 SD range of the control norms[10]. In some studies, MHE was diagnosed when both of the two tests were abnormal[1,29,30]. In others, MHE was diagnosed when at least one of the two tests was abnormal[24,31,32]. The present study compared PHES with NCT-A, NCT-B and DST for the diagnosis of MHE. The diagnosis of MHE on the basis of NCT-A and DST showed good agreement with PHES. If at least one of the NCT-A and DST tests was abnormal, MHE could be diagnosed with a sensitivity of 76.9% and a specificity of 96.3% with respect to PHES (AUC = 0.866, K = 0.735). Based on our study, we conclude that NCT-A and DST, which can be completed in minutes, are simple tools for screening MHE among Chinese inpatients with liver cirrhosis.

In summary, the preliminary normal values for all five tests of PHES in Chinese healthy volunteers have been constructed and are influenced by age and educational level. On the basis of a PHES score lower than -4, MHE was detected in 49.1% of the Chinese inpatients with liver cirrhosis. The combination of NCT-A and DST might be a simple and useful tool for the diagnosis of MHE in China.

COMMENTS

Background

Minimal hepatic encephalopathy (MHE) is widely prevalent in patients with cirrhosis. MHE is associated with impaired health-related quality of life, driving capability and can predict the development of overt hepatic encephalopathy. MHE is not detectable by routine physical or neurological examinations, and a specific neuropsychological/neurophysiological test is needed.

Research frontiers

International consensus recommends use of the psychometric hepatic encephalopathy score (PHES) for diagnosing MHE. The PHES has been standardized in Germany, Italy, Spain, India and Korea, but not in China.

Innovations and breakthroughs

This study constructed normal values for the PHES test in healthy Chinese volunteers and evaluated the usefulness of PHES for the diagnosis of MHE in Chinese patients with liver cirrhosis. In the present study, approximately 49% of patients with liver cirrhosis were classified as MHE. Compared to PHES, NCT-A and DST were able to diagnose MHE with a sensitivity of 76.9% and a specificity of 96.3% (AUC = 0.866, K = 0.735).

Applications

The results of the five neuropsychological tests of PHES are influenced by age and educational status. Age- and education-corrected nomograms can be used for MHE screening in patients with liver cirrhosis. The proportion of patients with MHE is associated with the severity of liver function. NCT-A and DST are simple and useful tools for the diagnosis of MHE in China.

Peer review

This is a single-center study from China aiming to validate the use of the PHES for the diagnosis of MHE in cirrhotic patients without overt hepatic encephalopathy. The study, which has created age- and education level-corrected values for the Chinese population, will enable other authors to diagnose MHE in patients with liver cirrhosis and to evaluate interventions.

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BRIEF ARTICLE

Perirenal space blocking restores gastrointestinal function in patients with severe acute pancreatitis

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Abstract

AIM: To investigate effects of perirenal space blocking (PSB) on gastrointestinal function in patients with severe acute pancreatitis (SAP).

METHODS: Forty patients with SAP were randomly allocated to receive PSB or no PSB (NPSB). All the SAP patients received specialized medical therapy (SMT). Patients in the PSB group received PSB + SMT when hospitalized and after diagnosis, whereas patients in the NPSB group only received SMT. A modified gastrointestinal failure (GIF) scoring system was used to assess the gastrointestinal function in SAP patients after admission. Pain severity (visual analog scale, 0 to 100) was monitored every 24 h for 72 h.

RESULTS: Modified GIF score decreased in both groups during the 10-d study period. The median score decrease was initially significantly greater in the PSB group than in the NPSB group after PSB was performed. During the 72-h study period, pain intensity decreased in both groups. The median pain decrease was significantly greater in the PSB group than in the NPSB group at single time points. Patients in the PSB group had significantly lower incidences of hospital mortality, multiple organ dysfunction syndrome, systemic inflammatory response syndrome, and pancreatic infection, and stayed in the intensive care unit for a shorter duration. However, no difference in terms of operation incidence was found between the two groups.

CONCLUSION: PSB could ameliorate gastrointestinal dysfunction or failure during the early stage of SAP. Moreover, PSB administration could improve prognosis and decrease the mortality of SAP patients.

Key words: Perirenal space blocking; Therapeutics; Severe acute pancreatitis; Gastrointestinal function; Prognosis

Core tip: This work aims to investigate the effects of perirenal space blocking (PSB) on the gastrointestinal function and clinical outcome of patients with severe acute pancreatitis (SAP). Our results showed that PSB could commendably improve the gastrointestinal dysfunction or failure during the early stage of severe SAP. Moreover, PSB administration could improve prognosis and significantly decrease the hospital mortality of SAP patients.

Sun JJ, Chu ZJ, Liu WF, Qi SF, Yang YH, Ge PL, Zhang XH, Li WS, Yang C, Zhang YM. Perirenal space blocking restores gastrointestinal function in patients with severe acute pancreatitis. *World J Gastroenterol* 2013; 19(46): 8752-8757 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i46/8752.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i46.8752

INTRODUCTION

Severe acute pancreatitis (SAP) has two major clinical stages, early and late. The first (early) stage is characterized by systemic inflammatory response syndrome (SIRS) and lasts for 10 d, whereas the second (late) stage is characterized by infectious complications, which account for most deaths in late-stage SAP patients[1-3]. SAP patients present symptoms of flatulence, abdominal distention, nausea, and vomiting related to a disturbance in gastrointestinal motility. Bacterial overgrowth in the ileus plays a major role in the pathogenesis of pancreatic infection[4-7]. Therefore, amelioration of intestinal dysmotility and stasis during the early period of SAP is important in reducing the risks associated with serious complications. Recent studies show that early enteral nutrition led to significantly lower incidences of multiple organ dysfunction syndrome (MODS), SIRS and pancreatic infection, and relieved intestinal dysmotility[8]. Nevertheless, early enteral nutrition is not usually practiced in SAP patients presenting disturbed gastrointestinal motility[9].

Gastrointestinal tract motor dysfunction in a pathological state is probably associated with muscular and neural dysfunction. For this reason, some researchers considered using epidural anesthesia therapy, which can shorten the duration of the postoperative intestinal paralysis, for patients with early-stage SAP[10,11]. Peridural anesthesia is also suggested by researchers but this therapy may not be applicable in all patients, and no rigorous, prospective controlled trials have been able to establish this therapy as a recommended treatment option[12].

The perirenal space is filled with fat. In acute pancreatitis, the perirenal fat and the bridging septa can be involved in the direct spread of inflammation[13,14]. This conclusion shows the direct relationship between perirenal space and the peripancreatic area. During SAP, an inflammatory exudate containing pancreatic enzymes leaks out from the pancreas, and its action of dissolving tissue inevitably stimulates the rich splanchnic ganglia and plexuses around the pancreas, which causes adverse reactions and reflection in the visceral nervous system and a series of pathophysiological disorders in the viscera, including gastrointestinal tract motor dysfunction. Considering the physiological and anatomical characteristics of the splanchnic nerves and the pancreas, and the pathological characteristics of SAP, the effect of perirenal space blocking (PSB) of a visceral nerve in the pancreatic region using 1% lidocaine on SAP treatment is studied. A simple, low-cost technique that could lead to short-term hospitalization or clinical treatment will be obtained.

MATERIALS AND METHODS

Study design

This is a single-center, prospective, and randomized controlled clinical trial. Patients randomly received either PSB or no PSB (NPSB) upon admission.

Table 1 The modified gastrointestinal failure score

Item	Points		
	0	1	2
Number of FI symptoms	None	1–2	⩾ 3
IAP (mmHg)	12	12–20	> 20 or ACS
Endotoxin concentration (pg/mL)	< 10	10–50	> 50
Computed tomography findings	None	Bowel wall thickening or intestinal extension	Bowel wall thickening and intestinal extension

FI: Food intolerance; IAP: Intra-abdominal pressure; ACS: Abdominal compartment syndrome.

Patients

All adult SAP patients (n = 40) admitted within 3 d after the onset of symptoms to the Department of General Surgery, the First Affiliated Hospital of Henan University of Science and Technology, from January 2012 to March 2013 were included in this study. SAP was defined as the presence of one or more local complications (*e.g.*, pseudocyst, necrosis or abscess) and/or organ failure, and acute physiology and chronic health evaluation APACHE Ⅱ score > 8 according to the widely used Atlanta criteria formulated in 1992[15]. The following criteria were used to exclude patients from the treatments: age (18 years old and below, or older than 75 years), pregnancy, evidence of malignancy, known cardiac morbidity including arrhythmia, severe pre-existing liver or kidney disease, leukopenia, allergic asthma, and known allergies. All the SAP patients received specialized medical therapy (SMT) for SAP[16], such as intensive monitoring, oxygen administration, fluid resuscitation, cessation of oral feeding, exocrine pancreatic suppression, and antibiotic prophylaxis. Patients in the PSB group received PSB + SMT upon hospitalization, whereas patients in the NPSB group only received SMT after a definite diagnosis. This study was conducted in accordance with the declaration of Helsinki, with approval from the Ethics Committee of the First Affiliated Hospital of Henan University of Science and Technology. Written informed consent was obtained from all participants or their first-degree relatives.

Evaluation protocol for gastrointestinal function

A modified gastrointestinal failure (GIF) scoring system was used to assess the gastrointestinal function in SAP patients. The system combined food intolerance (FI) symptoms, intra-abdominal pressure (IAP), endotoxin concentration and computed tomography findings into a 3-grade score, which is the modified GIF score (Table 1)[17].

PSB

One Teflon epidural catheter was placed for intermittent perirenal space blockade under local anesthesia after a definite diagnosis. An epidural transfixion pin was used to puncture the right lumbar region of SAP patients and was positioned into the right capsule of the kidney by the vectoring of B-mode ultrasonic diagnostic equip-

ment. Subsequently, the catheter was placed within the perirenal space through the transfixion pin. The external end of the catheter was fixed to the skin of the lumbar region. The patients were allowed to recover by normal and calm breathing, and lidocaine (100 g/L, 0.08 L/8 h) was intermittently injected into the capsule through the catheter. This regimen was administered for 10 d for the PSB group immediately after the diagnosis, and before randomization.

Data collection

Upon admission, we recorded the baseline data, including age, gender, etiology, diagnoses, and whether SIRS had been diagnosed. The APACHE Ⅱ scores[18] were recorded daily for 1-3 d. C-reactive protein (CRP) level, and serum endotoxin concentration[19] were recorded 1, 3, 7 and 10 d after admission. According to the manufacturer's instructions, we measured serum endotoxin with Gram-negative endotoxin detection reagents (Beijing Gold Mountainriver Technology Development Corporation Limited, China). Contrast-enhancement computed tomography (CECT) was performed 1, 3, 7 and 10 d after admission and the computed tomography severity index[20] score was calculated thereafter. We also assessed the image of the gastrointestinal tract. IAP was measured using the bladder technique, according to the method recommended by the World Society of Abdominal Compartment Syndrome in 2006[21]. For the duration of hospital stay, we recorded the gastrointestinal functional rehabilitation time (including venting and defecation time), the number of patients that received operation, and the number of patients whose clinical course was complicated by systemic and local complications such as MODS or pancreatic infection. The hospital mortality and length of stay were also recorded. We evaluated the gastrointestinal function of the two groups of SAP patients using the modified GIF score upon admission and for the next 3, 7 and 10 d. Patient abdominal pain was recorded daily using a standard visual analog scale (VAS) ranging from 0 ("no pain") to 100 ("unbearable pain")[22].

Definitions

The following criteria were used to diagnose pancreatic infection: positive bacterial culture of peripancreatic fluid and repeated increases in body temperature[3,23]. IAH was defined by a sustained or repeated pathological elevation in IAP ⩾ 12 mmHg[21]. Abdominal compartment syndrome was defined as a sustained IAP > 20 mmHg associated with new organ dysfunction/failure[21]. MODS was defined as the dysfunction of two or more organs. Bowel wall thickening was defined as thickness of 3 mm or greater on CECT, and intestinal extension was defined as a dilatation of more than 2.5 cm on CECT[24,25]. Enteral feeding started as early as possible, if the patient had no obvious contraindications such as ileus or intestinal bleeding. FI was diagnosed when enteral feeding was unsuccessful and had to be discontinued because of repeated nausea, vomiting, high gastric residual volume, abdominal pain or distension, and diarrhea[19,26,27]. We counted the frequency of signs for every patient as the number of symptoms of food intolerance.

Table 2 Patient characteristics upon admission *n* (%)

	PSB group (*n* = 20)	NPSB group (*n* = 20)	*P* value
Age (yr)	43 (34.5-55)	45 (35-60)	0.589
Sex (male: female)	11:9	12:8	0.749
Etiology			
Biliary origin	10 (50)	11 (55)	0.752
Hyperlipidemia	7 (35)	6 (30)	0.736
Alcohol abuse	2 (10)	1 (5)	0.548
Idiopathic	1 (5)	2 (10)	0.548
BMI	24.6 (23.5-26.8)	25.8 (23.9-28.8)	0.158
APACHEII score	9.5 (8.5-11)	10 (8-11.5)	0.994
CRP (mg/L)	203.5 (188-253)	195 (161-247.5)	0.214
Pain > 77 mm (VAS)	13	15	0.654

PSB: Perirenal space blocking; NPSB: No perirenal space blocking; BMI: Body mass index; VAS: Visual analogue scale; CRP: C-reactive protein.

Statistical analysis

All the data are presented as median (interquartile range) if not stated otherwise. Categorical variables are expressed as absolute numbers or in percentages, and were analyzed using the χ^2 test. Continuous variables were compared by the Mann-Whitney *U* test or Wilcoxon signed-rank test, as appropriate. Statistical package for the social sciences (SPSS, version 17.0, Chicago, IL, United States) software was used for statistical analyses. $P < 0.05$ was considered statistically significant.

RESULTS

Baseline data of patients

There were no significant differences between the 2 groups with regard to sex distribution, age, body weight, or cause of pancreatitis. The severity of pain, acute physiology and chronic health evaluation APACHE Ⅱ, and serum CRP did not significantly differ between the two groups. The demographic data and clinical parameters of the patients upon admission are presented in Table 2.

Effect on pain

During the 72-h study period, pain intensity decreased in both groups. VAS data were depicted as median values (ranges) for the evaluation of pain intensity at specific time points. The median pain decrease (VAS) was significantly greater in the PSB group (-53) than in the NPSB group (-23) at 24 h; -67 than -46 at 48 h; and -76 than -49 at 72 h. Thus, the magnitude of median pain relief was better in the PSB group compared with the NPSB group (Table 3).

GIF score

During the 10-d study period, modified GIF score decreased in both groups, from 4.56 to 1.00 in the PSB

Table 3 Pain intensity between two groups

	Pain severity:	Pain severity: Change from baseline (ΔVAS)		
	Baseline (VAS)	At 24 h	At 48 h	At 72 h
PSB group (n = 20)	78	-53	-67	-76
NPSB group (n = 20)	77	-23	-46	-49
P value	1.000	0.005	0.018	0.025

PSB: Perirenal space blocking; NPSB: No perirenal space blocking; VAS: Visual analogue scale.

Table 4 Modified gastrointestinal failure score variables between two groups

	Before PSB performed	Hospital day			
		1 d	3 d	7 d	10 d
PSB group (n = 20)	4.56	2.6	2.12	1.43	1.000
NPSB group (n = 20)	4.34	3.98	3.56	2.58	2.13
P value	1.000	0.042	0.025	0.031	0.012

PSB: Perirenal space blocking; NPSB: No perirenal space blocking.

group and from 4.34 to 2.13 in the NPSB group. The median score decrease was initially significantly greater in the PSB group than in the NPSB group (P = 0.042) after hospitalization for 24 h (PSB was performed as soon as PSB group patients were admitted). The variance tendency of the modified GIF score in the two groups is presented in Table 4.

Comparison of outcome variables between the two groups

As presented in Table 5, patients in the PSB group had significantly lower incidences of hospital mortality, MODS, SIRS, pancreatic infection and shorter intensive care unit stay during hospital stay. However, no difference in terms of operation incidence was found between the two groups.

DISCUSSION

The celiac plexus is a major interchange for autonomic fibers, receiving many of the thoracic splanchnic nerve fibers as they course toward the abdominal organs. Pain associated with pancreatic morbidity is intense and severe, and for many years, the celiac plexus has been a target for pain block treatments[28]. The celiac plexus lies in front of the aorta at the level of the celiac trunk[29]. It is composed of a dense network of sympathetic nerve fibers that travel in parallel to the anterior surface of the abdominal aorta and the origin of the celiac artery. The celiac plexus transmits neural signals originating from all abdominal viscera and the majority of pelvic viscera, including the pancreas, liver, gallbladder, stomach, renal pelvis, ureter, and intestine proximal to the transverse colon[30].

Both the pancreas and kidney are retroperitoneal organs and are adjacent to each other. In the retroperitoneal

Table 5 Clinical outcome variables *n* (%)

	NPSB group (n = 20)	PSB group (n = 20)	P value
Hospital mortality	6 (30)	1 (5)	0.037
ICU stay (d)	12 (8-21)	9 (5-14)	0.033
Pancreatic infection	8 (40)	2 (10)	0.028
MODS	9 (45)	3 (15)	0.038
SIRS	14 (70)	7 (35)	0.027
Surgical operation	4 (20)	2 (10)	0.376

ICU: Intensive care unit; MODS: Multiple organ dysfunction syndrome; SIRS: Systemic inflammatory response syndrome; PSB: Perirenal space blocking; NPSB: No perirenal space blocking.

space, the left and right kidneys and their adipose capsules are next to the pancreas, celiac artery, and superior mesenteric artery root. Thorntons' findings show that the perirenal spaces communicate with each other across the midline, and with the pelvic extraperitoneal spaces. Clinical implications include the potential flow of perinephric collections into the pelvis or across the midline[31]. This means that the celiac ganglion and plexus, including the plexus pancreaticus, and the renal and superior mesenteric plexuses, are located in the gallery of bilateral perirenal spaces. During SAP, an inflammatory exudate containing pancreatic enzymes leaks out from the pancreas and its action of dissolving tissue inevitably stimulates the rich splanchnic ganglia and plexuses around the pancreas, which causes adverse reactions and reflection in the visceral nervous system and a series of pathophysiological disorders in the viscera, including gastrointestinal tract motor dysfunction.

Considering the physiological and anatomical characteristics of the splanchnic nerves and the pancreas, and the pathological characteristics of SAP, the effect of PSB of a visceral nerve in the pancreatic region using 1% lidocaine on SAP treatment was studied .

Nutrition support is important in the adjunctive management of SAP patients. Meta-analysis shows that in patients with acute pancreatitis, total parenteral nutrition significantly increases the risk of infective complications, the likelihood of a surgical intervention (to control pancreatic infection) and the length of hospital stay, compared with enteral nutrition[32]. Nevertheless, early enteral nutrition is not usually practiced in SAP patients presenting disturbed gastrointestinal motility[9].

This clinical study investigated the effects of PSB on the gastrointestinal function and on the clinical outcome of SAP patients. We found that PSB could commendably ameliorate gastrointestinal dysfunction or failure during the early stage of SAP. Moreover, PSB administration could improve prognosis and significantly decrease the hospital mortality of SAP patients.

Recent studies have shown that early enteral nutrition led to significantly lower incidences of MODS, SIRS and pancreatic infection, and relieved intestinal dysmotility[8]. Gastrointestinal tract motor dysfunction in a pathological state is probably associated with muscular and neural dys-

function. For this reason, some researchers considered using epidural anesthesia therapy, which can shorten the duration of the postoperative intestinal paralysis[11], for the patients with early-stage SAP[10]. In fact, the beneficial effect of epidural anesthesia has been attributed to blockade of a sympathetic nerve, which contributes to the recovery of gastrointestinal tract motor function[33]. Peridural anesthesia has also been suggested previously, but this may not be applicable in all patients and no rigorous, prospective controlled trials have been able to establish this therapy as a recommended treatment option[12]. Epidural anesthesia can selectively block sympathetic nerve fibers which supply the pancreas, but in clinical practice, this technique is difficult to implement because of the different effects of anesthesia in individuals and the different classes of nerve fibers. The risks include total spinal anesthesia, blood circulation disorders, respiratory inhibition, deep venous thrombosis, and bedsore. For the patients with SIRS, this method may lead to fatal complications such as intraspinal hematoma, and intraspinal infection. PSB can prevent these problems because of its common use for different treatments, including acute anuria, paralytic ileus, stomach cramps, bronchial asthma, postoperative abdominal distension, and burn shock. In clinical work, the technique of PSB is common, safe, simple, low-cost, exempt from B ultrasound guidance, and easy to implement in all hospitals. Furthermore, the manual operation is easy to replicate. There are several limitations in this study. Due to the small sample size and the single-center design, our results might be insufficient to reach a definite conclusion. Therefore, the accuracy should be tested further using a larger sample size. Moreover, since this study was not based on a pathophysiological model, the precise mechanisms of PSB in SAP patients should be verified by more basic experiments.

In conclusion, PSB could commendably ameliorate gastrointestinal dysfunction or failure during the early stage of SAP. Moreover, PSB administration could improve prognosis and significantly decrease the hospital mortality of SAP patients. However, the precise mechanisms of PSB for SAP are still not clear, and further studies are required to verify our conclusions.

COMMENTS

Background

Severe acute pancreatitis (SAP) has two major clinical stages, early and late. The first (early) stage is characterized by systemic inflammatory response syndrome (SIRS) and lasts for 10 d, whereas the second (late) stage is characterized by infectious complications, which account for most deaths in late-stage SAP patients

Research frontiers

The paper is for the first time investigated the effects of perirenal space blocking (PSB) on gastrointestinal function in patients with SAP.

Innovations and breakthroughs

PSB could ameliorate gastrointestinal dysfunction or failure during the early stage of SAP. Moreover, PSB administration could improve prognosis and decrease the mortality of SAP patients.

Peer review

It is a good study, which showed that PSB was associated with a significant decrement of pain, hospital mortality, multiple organ dysfunction syndrome, SIRS and pancreatic infection in patients with SAP.

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BRIEF ARTICLE

Association between *TNF-α* and *IL-1β* genotypes *vs* *Helicobacter pylori* infection in Indonesia

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Abstract

AIM: To investigate the correlation between the *Helicobacter pylori* (*H. pylori*) infection and host genetic background of healthy populations in Indonesia.

METHODS: In March 2007, epidemiological studies were undertaken on the general population of a city in Indonesia (Mataram, Lombok). The participants included 107 men and 187 women, whose ages ranged from 6 to 74 years old, with an average age of 34.0 (± 14.4) (± SD). The *H. pylori* of subject by UBT method determination, and through the polymerase chain reaction with confronting two-pair primers (PCR-CTPP) method parsing the single nucleotide polymorphism of interleukin (IL)-8, IL-4, IL-1β, CD14, tumor necrosis factor (TNF-α) and tyrosine-protein phosphates non-receptor type 11 (PTPN11) genotypes. The experimental data were analyzed by the statistical software SAS.

RESULTS: The *H. pylori* infection rates in the healthy Indonesian population studied were 8.4% for men and 12.8% for women; no obvious differences were noted for *H. pylori* infection rates by sex or age. TC genotypes of IL-4, TC and CC genotypes of TNF-α, and GA genotypes of PTPN11, were higher in frequency. Both CC and TC genotype of TNF-α T-1031C loci featured higher expressions in the healthy Indonesian population Indonesia studied of (OR = 1.99; 95%CI: 0.67-5.89) and (OR = 1.66; 95%CI: 0.73-3.76), respectively. C allele of IL-1β T-31C gene locus was at a higher risk (OR = 1.11; 95%CI: 0.70-1.73) of *H. pylori* infection, but no statistical significance was found in our study.

CONCLUSION: We reveal that the association between the TNF-α and IL-1β genotypes may be the susceptibility of *H. pylori* in the studied population.

Key words: *Helicobacter pylori*; Tumor necrosis factor; Interleukin-1β; Infection; Allele

Core tip: We found single nucleotide polymorphism of tumor necrosis factor-α and allele of interleukin-1β having high frequency in the healthy Indonesian population, which may be associated with potential contact with *Helicobacter pylori* (*H. pylori*) infection. Throughout, *H. pylori* studies were conducted in patients, and treatment was based on quadruple antibiotics to eradicate *H. pylori* infection in clinical trials. However, the implications of the individual differences in recurrent infections and drug resistance of *H. pylori* and other issues must be addressed. Therefore, vaccine development for prevention of *H. pylori* will be a topical issue in the coming years.

Zhao Y, Wang JW, Tanaka T, Hosono A, Ando R, Tokudome S, Soeripto, Triningsih FXE, Triono T, Sumoharjo S, Achwan EYWA, Gunawan S, Li YM. Association between *TNF-α* and *IL-1β* genotypes *vs Helicobacter pylori* infection in Indonesia. *World J Gastroenterol* 2013; 19(46): 8758-8763 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i46/8758.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i46.8758

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a microaerophilic (G-) bacteria that colonizes the area of stomach and duodenum, causing chronic inflammation of the gastric mucosa, the development of the stomach ulcers and even gastric cancer[1]. *H. pylori* is a class I carcinogen, and has been identified by the WHO as a cancer-causing prokaryotes[2]. More than 50% of the world's population infected with *H. pylori*, but 80% of people infected with *H. pylori* show no symptoms[3]. The *H. pylori* infection occurs mainly in economically underdeveloped regions, and the *H. pylori* infection rates of China, Japan and Korea were higher than developed countries[4-6], while the infection rates of Thailand and Vietnam were higher than Indonesia in Southeast Asia[7]. Regarding the ethnic groups of Singapore, *H. pylori* infection and the incidence of digestive diseases was higher in the Indian and Chinese than the Malay population[8]. The above studies have shown that *H. pylori* infection and geographical, ethnic and host genetic background is relational, and that the bacteria play a key role in the development of gastric cancer.

Single nucleotide polymorphism (SNP) is caused by a single nucleotide mutation in the genomic level DNA sequence polymorphisms. It is the most common type of genetic variation in humans. Accounting for more than 90% of all known polymorphisms, SNP is widespread in the human genome and there is a close relationship between the incidences of the disease[9]. Interleukin-8 (IL-8) is an important regulatory factor in the development of gastritis for *H. pylori* associated infection[10]. The interleukin-4 (IL-4) promotes HLA class Ⅱ antigen expression in B-cells[11], and IL-1β protein is an important inflammatory mediator, involved in infected *H. pylori* of the stomach inflammation reaction[12,13]. Have a study reported that the cluster of differentiation 14 (CD14) is an important receptor in the submission of *H. pylori* lipopolysaccharide (LPS). The relationship is between CD14 with the weakening of the immune response in the body to LPS of *H. pylori* and to reduce the proinflammatory cytokine secretion levels[14]. Tumor necrosis factor-α (TNF-α) is involved in inflammation, immune regulation and tissue repair, and the TNF-α is an important factor in the development of digestive diseases[15]. The tyrosine-protein phosphates non-receptor type 11 (PTPN11) gene is located in chromosome 12, and it has been found that the expression product of SHP-2 to participate in the cytotoxin-associated protein A (cagA) deformation caused by gastric epithelial cells eventually causes gastric cancer[16].

The purpose of this study was to investigate the correlation of *H. pylori* infection in a healthy Indonesian population and host genetic background, and to reveal susceptibility genes of *H. pylori*, as well as new strategies for the prevention and treatment of gastric cancer.

MATERIALS AND METHODS

Study population

In recent years, we have conducted long-term international cooperation in research, exploring the impact of environmental factors on the risk factors of gastric cancer in Southeast Asia, including the countries of Thailand, Vietnam and Indonesia, as well as Gansu Province in China. In March 2007, epidemiological studies were undertaken on the general population of a city in Indonesia (Mataram, Lombok). The participants included 107 men and 187 women, whose ages ranged from 6 to 74 years old, with an average age of 34.0 (± 14.4) (± SD). We detected and analyzed the *H. pylori* of the observation target as well as the genetic background of the host, namely the *IL-8*, *IL-4*, *IL-1β*, *CD14*, *TNF-α* and *PTPN11* genotypes. All the subjects' informed consent was approved by the Nagoya City University Graduate School of Medical Ethics Committee.

Urea breath test

H. pylori infection was determined by UBT, UBiT-IR300 kits (Otsuka Pharmaceutical Co., Tokyo, Japan) with ⩾ 2.5‰ considered as positive. All subjects were classified as *H. pylori* -positive (+) or -negative (-) in this study[7,8].

Genotyping of DQA1 and DQB1

A template of genomic DNA was isolated from 100 μl of peripheral blood leukocytes by the Nucleic Acid Purification System (MagExtractor MFX-6000 TOYOBO, Japan). We carried out a single nucleotide polymorphism (SNP) analysis of the *IL-8*, *IL-4*, *IL-1β*, *CD14*, *TNF-α* and *PTPN11* genotypes by two pairs of polymerase chain reaction (PCR-CTPP)[17-21].

Table 1 *Helicobacter pylori* infection by sex and age in Indonesian people *n* (%)

Indonesia	*H. pylori* (+) *n* = 33	*H. pylori* (-) *n* = 261
Sex		
Male	9 (8.4)	98 (91.6)
Female	24 (12.8)	163 (87.2)
Age, yr		
⩽ 30	12 (9.6)	113 (90.4)
31-40	9 (11.5)	69 (88.5)
41-50	7 (14.6)	41 (85.4)
51-60	3 (9.4)	29 (90.6)
⩾ 60	2 (18.2)	9 (81.8)
Mean age, yr	36.3 ± 14.6 (SD)	33.7 ± 14.4 (SD)

H. pylori: *Helicobacter pylori*.

Statistical analysis

Differences in distribution by age according to prevalence of *H. pylori* infection were examined by *t*-test, while differences in distribution by sex and genotype were assessed with a Chi-square test. Hardy-Weinberg equilibrium was examined for *IL-8*, *IL-4*, *IL-1β*, *CD14*, *TNF-α* and *PTPN11* gene polymorphisms. Multi-comparisons for *IL-8*, *IL-4*, *IL-1β*, *CD14*, *TNF-α* and *PTPN11* genotypes were made according to the Bonferroni method. Associations of the *IL-8*, *IL-4*, *IL-1β*, *CD14*, *TNF-α* and *PTPN11* genotypes and SNP with *H. pylori* infection were examined by OR and 95%CI using unconditional logistic regression analysis. Statistical significance was determined as $P < 0.05$. All the statistical analyses were performed using the SAS software package (version 9.1).

RESULTS

The positive *H. pylori* infection rate as a whole was 11.2% in Mataram (Table 1). No obvious differences were noted for *H. pylori* infection rates by sex or age. TC genotypes of IL-4, TC and CC genotypes of TNF-α, and GA genotypes of PTPN11 were frequent. Individuals carrying TC and CC allele of TNF-α was noted to be at higher risk of *H. pylori* infection, compared with those carrying TT allele of TNF-α (OR = 1.66, 95%CI: 0.73-3.76) and (OR = 1.99, 95%CI: 0.67-5.85), We also found TT and CT genotypes of CD14 C-159T (OR = 1.09, 95%CI: 0.37-3.20) and (OR = 1.26, 95%CI: 0.50-3.19), but no statistical significance was found in our study (Table 2). We found C allele had a higher frequency than T allele of IL-1β genotype in the studied population (OR = 1.11, 95%CI: 0.70-1.73), but again no statistical significance was found (Table 3).

DISCUSSION

In 50% of the world's population was infected *H. pylori* infection rates in developing countries were higher than in developed countries, and it has been reported that hosts at an early age have been infected[22]. Indonesia, located in Southeast Asia, is a developing country, but

Table 2 Association between *Helicobacter pylori* infection and interleukin 1β, interleukin 4, interleukin 8, CD14, tumor necrosis factor-α, tyrosine-protein phosphates non-receptor type 11 single nucleotide polymorphism in Indonesian people *n* (%)

Polymorphism	*H. pylori* (+) *n* = 33	*H. pylori* (-) *n* = 261	OR[1]	95%CI[1]
IL-1β T-31C				
TT	8 (24.2)	59 (22.6)	ref	
CC	8 (24.2)	73 (28.0)	0.82	0.29-2.32
TC	17 (51.5)	129 (49.4)	1.05	0.42-2.59
TC/CC	25 (75.8)	202 (77.4)	0.96	0.41-2.26
IL-4 T-33C				
TT	15 (45.5)	128 (49.0)	ref	
CC	2 (6.1)	19 (7.3)	0.83	0.17-3.99
TC	16 (48.5)	114 (43.7)	1.24	0.58-2.64
CC/TC	18 (54.5)	133 (51.0)	1.18	0.56-2.45
IL-8 T-251A				
TT	14 (42.4)	98 (37.6)	ref	
AA	8 (24.2)	49 (18.8)	1.25	0.48-3.27
TA	11 (33.3)	114 (43.7)	0.74	0.32-1.72
TA/AA	19 (57.6)	163 (62.5)	0.89	0.42-1.88
CD14 C-159T				
CC	7 (21.2)	65 (24.9)	ref	
TT	8 (24.2)	65 (24.9)	1.09	0.37-3.20
CT	18 (54.6)	131 (50.2)	1.26	0.50-3.19
CT/TT	26 (78.8)	196 (75.1)	1.2	0.50-2.92
TNF-α T-1031C				
TT	11 (33.3)	120 (46.0)	ref	
CC	6 (18.2)	36 (13.8)	1.99	0.67-5.89
TC	16 (48.5)	105 (40.2)	1.66	0.73-3.76
CC/TC	22 (66.7)	141 (54.0)	1.74	0.80-3.76
PTPN11 G/A at intron 3				
GG	17 (51.5)	151 (57.9)	ref	
AA	1 (3.0)	17 (6.5)	0.6	0.07-4.86
GA	15 (45.5)	93 (35.6)	1.49	0.70-3.15
GA/AA	16 (48.5)	110 (42.2)	1.37	0.65-2.85

[1]Odds rate with CI adjusted for age and sex by logistic regression model. *H. pylori*: *Helicobacter pylori*; IL: Interleukin; PTPN11: Tyrosine-protein phosphates non-receptor type 11.

we found that the country has an *H. pylori* infection rate which was very low. We investigated associations between SNP of the host *IL-8*, *IL-4*, *IL-1β*, *CD14*, *TNF-α* and *PTPN11* gene polymorphisms and *H. pylori* prevalence in an Indonesian population with an *H. pylori* infection rate of 11.2% in people residing in Mataram, Lombok Island. Although SNP of host *IL-8*, *IL-4*, *IL-1β*, *CD14*, *TNF-α* and *PTPN11* genotype with *H. pylori* infection were not found to have statistical significance in our study, we saw that an observation target who had the CC and TC genotype of *TNF-α* gene were at a higher risk of contracting *H. pylori* infection. Perhaps, *TNF-α* gene plays a key role in the *H. pylori* infection process.

H. pylori is widely present in the environment, and it can be isolated in surface waters[23], *i.e.*, transmitted by the fecal - oral route[24]. Studies have shown that through certain digestive diseases and strains of *H. pylori*, Cytotoxin-associated protein A (cagA) is now known as the most important virulence factors of *H. pylori*[25]. CagA is an *H. pylori* cag poison island (cag-PAI) flag, and by cag-PAI coded protein is composed of a bacterial type Ⅳ secretion system into gastric epithelial cells, which ultimately

Table 3 Association between *Helicobacter pylori* infection and allele of interleukin 1β, interleukin 4, interleukin 8, CD14, tumor necrosis factor-α, tyrosine-protein phosphates non-receptor type 11 in Indonesian people *n* (%)

Allele	*H. pylori* (+) *n* = 66	*H. pylori* (-) *n* = 522	OR[1]	95%CI
IL-1β T-31C				
T	33 (50.0)	247 (47.3)	ref	
C	33 (50.0)	275 (52.7)	1.10	0.70-1.73
IL-4 T-33C				
T	46 (69.7)	370 (70.9)	ref	
C	20 (30.3)	152 (29.1)	0.95	0.58-1.56
IL-8 T-251A				
T	39 (59.1)	310 (59.4)	ref	
A	27 (40.9)	212 (40.6)	0.99	0.62-1.57
CD14 C-159T				
C	32 (48.5)	261 (50.0)	ref	
T	34 (51.5)	261 (50.0)	0.95	0.60-1.49
TNF-α T-1031C				
T	38 (57.6)	345 (66.1)	ref	
C	28 (42.4)	177 (33.9)	0.73	0.46-1.15
PTPN11 G/A at intron 3				
G	49 (74.2)	395 (75.7)	ref	
A	17 (25.8)	127 (24.3)	0.94	0.56-1.57

[1]Odds rate with CI adjusted for age and sex by logistic regression model. *H. pylori*: *Helicobacter pylori*; IL: Interleukin; PTPN11: Tyrosine-protein phosphates non-receptor type 11.

causes gastric mucosal epithelium, the morphological changes of the cells and the formation of a humming-bird-like structure[26]. Host infected cagA-positive *H. pylori* is less likely to cause digestive diseases, but may damage the gastric mucosal barrier and is cagA related. *H. pylori* infection with strains, geographical, ethnic, and environmental and host genetic background was a correlation.

The IL-8 as a neutrophil chemoattractant and activating factor, which relates to *H. pylori* infection, resulting in second messenger of the mucosal inflammatory response in the *H. pylori* pathogen city, plays an important role of intermediary. But what components of *H. pylori* surface play a major role in the induction of IL-8 expression is still one of the main points about *H. pylori* pathogenesis. Of *H. pylori* cytotoxin-associated protein (cagA) and vacuolating cytotoxin (vacA) on gastric epithelial IL-8 secretion, showing expression of cagA and vacA *H. pylori* strains (vacA+, cagA+) direct stimulation of gastric epithelial cell lines IL-8 mRNA expression and protein secretion of IL-8, suggests that expression of the gene product and cagA *H. pylori* strains induced gastric epithelial expression of IL-8 in the main factors[11]. In addition to *H. pylori* gastric epithelial cells directly stimulating the production of IL-8, the inflammation locally produced of TNF-α, transcription factor activation of the IL-1, was also an up-regulated expression of IL-8[11]. Furthermore *H. pylori*, in addition to the expression of IL-8 induced gastric epithelial cells, also stimulates gastric epithelial cells TNF-α, IL-1β expression[27,28]. In *H. pylori* infection, IL-8 chemotaxis of neutrophil infiltration and epithelial damage caused by *H. pylori* vacuoles toxins can promote mucous membrane endocytosis bacterial products and induction of mucosal phagocytic cells to secrete cytokines IL-1β, TNF-α and IL-8; neutrophils are attracted to the infected local, while neutrophils becomes the main source of iL-1, TNF-α and iL-8 induced inflammatory cytokines. Neutrophil elastase also relates to the epithelial cells induced by IL-8 gene expression, suggesting that the neutrophil enzyme release cytokines can induce a continuity of the inflammatory process itself[29], and *H. pylori*-induced IL-8, IL-1β cytokine expression throughout the entire *H. pylori* infection period[30]. Studies found that Protein-tyrosine phosphatase, non-receptor-Type11 (PTPN11) encoding Srchomology 2 domain-containing pro-Tein tyrosine phosphatase-2 (SHP-2) in CagA-induced gastric epithelial cell deformation, that eventually cause the gastric process, played a very important role, and the genetic background of the PTPN11 shows certain racial difference[31,32]. The IL-4 by CD4+ T cell subsets, B cells and mast cells secreted pleiotropic cytokines involved in inflammation, mucosal repair, cell proliferation and apoptosis and other physiological and pathological processes; changes in the expression levels may also affect pathogenesis of *H. pylori* infection, resulting in a host of different clinical results. The *H. pylori* infection caused by non-ulcerative gastritis can lead to local Th0 cells producing and secreting large amounts of cytokines IL-4; however, in patients with peptic ulcers, *H. pylori* infection can be caused by the polarization of Th1 cells[33]. Studies suggest that the CD14 gene C/T mutation may lead to the activation of the CD14 promoter enhanced transcription of the CD14 gene, while monocytes' high expression of CD14 and CD14 can regulate the secretion of LPS-induced IL-1 and TNF-α[15,34]. TNF-gene coding region mutations may affect TNF-α activity, caused by TNF-α allele or genetic type associated with *H. pylori* associated gastric duodenal disease susceptibility. In an infected *H. pylori* host of Japan, it was found that the genotype of the TNF-α-857 C/C and 1031 C/C group serological detection of *H. pylori* was the lowest positive rate, and in the TNF-α-857 T/T and TNF-B-1031 T/T genotype the serum *H. pylori* positive rate was the highest[35]. The C/C and T/C genotypes of TNF-α T-1031C locus were at the highest risk from *H. pylori* infection in our study.

The development of gastric cancer is a complex process, *H. pylori* infection is caused by one of the risk factors of gastric cancer. In addition, there are environmental factors, social factors, host genetic background and lifestyle. Directly use hand grasp to pilaf is very common, and the schistosome liver disease has also been often reported in Indonesia[36]; however, *H. pylori* infection and gastric cancer incidence rate were very low. In addition, have also been reports that complications of the esophagus caused by reflux esophagitis after sterilization of cancer have tended to increase[37]. And resistant strains of *H.pylori* by sterilization treatment have been reported[38]. Therefore, it appears that sterilization treatment is not the best means of prevention of gastric cancer. This study explored *H. pylori* infection with immune response

gene polymorphisms in a healthy Indonesian population. Although there was no statistical significance in SNP of *IL-8*, *IL-4*, *IL-1β*, *CD14*, *TNF-α* and *PTPN11* gene polymorphisms, we found SNP of TNF-αT-1031C locus was the highest risk of *H. pylori* infection. Our study provides the basis for future research data, and a new direction for the prevention of *H. pylori* infection.

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COMMENTS

Background

Helicobacter pylori (H. pylori) infection in developing countries is high in comparison with developed countries. Indonesia is a developing country located in Southeast Asia, but the prevalence of *H. pylori* in Indonesia is lower than other countries of Southeast Asia.

Innovations and breakthroughs

Throughout, *H. pylori* studies were conducted in patients, and treatment was based on quadruple antibiotics to eradicate *H. pylori* infection in clinical settings. However, the implications of the individual differences in recurrent infections and drug resistance of *H. pylori* and other issues must be addressed. The authors observed that the object was a healthy crowd, which reveals that in the host genetic background there is a certain association with *H. pylori* infection.

Applications

This study provided basic vaccine development data for the prevention of *H. pylori*, and for the prevention of gastric cancer through the advancement of new ideas.

Terminology

H. pylori is a Gram-negative, microaerophilic bacterium found in the stomach. It was identified in 1982 by the Australian scientists Barry Marshall and Robin Warren, who found that it was present in patients with chronic gastritis and gastric ulcers, conditions that were not previously believed to have a microbial cause. It is also linked to the development of duodenal ulcers and stomach cancer. However, over 80 percent of individuals infected with the bacterium are asymptomatic, and it has been postulated that it may play an important role in the natural stomach ecology.

Peer review

The manuscript is interesting, but the absence of statistical significance is an important issue.

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BRIEF ARTICLE

Silencing Bmi-1 enhances the senescence and decreases the metastasis of human gastric cancer cells

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Abstract

AIM: To evaluate the impact of Bmi-1 on cell senescence and metastasis of human gastric cancer cell line BGC823.

METHODS: Two pairs of complementary small hairpin RNA (shRNA) oligonucleotides targeting the Bmi-1 gene were designed, synthesized, annealed and cloned into the pRNAT-U6.2 vector. After DNA sequencing to verify the correct insertion of the shRNA sequences, the recombinant plasmids were transfected into BGC823 cells. The expression of Bmi-1 mRNA and protein was examined by reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting. The effects of Bmi-1 knockdown on cell senescence and metastasis were determined by the β-Gal activity assay and Boyden chamber assay, respectively.

RESULTS: The double-stranded oligonucleotide fragments of Bmi-1 short interfering RNA (siRNA) cloned into pRNAT-U6.2 vector conformed to the inserted sequence. RT-PCR and Western blotting indicated that the expression levels of Bmi-1 gene mRNA and protein were markedly decreased in transfected BGC823 cells with pRNAT-U6.2-si1104 and pRNAT-U6.2-si1356, especially in transfected BGC823 cells with pRNAT-U6.2-si1104, compared with two control groups (empty vector and blank group). In particular, Bmi-1 protein expression was almost completely abolished in cells transfected with the recombinant vector harboring shRNA targeting the sequence GGAGGAGGTGAATGATAAA (nt1104-1122). Compared with untransfected cells and cells transfected with the empty vector, the mean percentage of senescent cells increased and the number of cells passing through the Matrigel decreased in cells transfected with the recombinant vectors.

CONCLUSION: Silencing Bmi-1 by RNA interference can increase the senescent cell rate and effectively reduce the metastasis of gastric cancer cells.

Key words: Bmi-1; Gastric cancer; Senescence; Metastasis

Core tip: The overexpression of Bmi-1 contributes to the development of cancers. This study aimed at to evaluate the impact of Bmi-1 on the senescence and metastasis of human gastric cancer. The results demonstrated that inhibition of *Bmi-1* gene expression can enhance the senescence of human gastric cancer cells and inhibit the invasion and metastasis of gastric cancer. This research has provided an indication that Bmi-1 inhibitors might be developed as new agents for gastric cancer.

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senescence and decreases the metastasis of human gastric cancer cells. *World J Gastroenterol* 2013; 19(46): 8764-8769 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i46/8764.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i46.8764

INTRODUCTION

Bmi-1 (B lymphoma Mo-MLV insertion region 1 homolog), a member of the polycomb group (PcG), functions as a transcriptional repressor and presents with high expression in many tumors, indicating a poor prognosis[1,2]. Several lines of evidence suggest that Bmi-1 blocks cell senescence and proliferation[3,4], and the *Bmi-1* gene is also associated with tumor invasion and metastasis[5]. Based on a list of genes on a wild-type and Bmi-1-deficient genetic background, Bmi-1 has been identified as a predictor of the response to therapy and survival in multiple types of cancer[6,7]. Therefore, this study intended to silence Bmi-1 in BGC823 cells by RNA interference, to observe the role of Bmi-1 in the senescence and metastasis of gastric cancer cells.

MATERIALS AND METHODS

Materials

Short interfering RNA (siRNA) vector pRNAT-U6.2 was purchased from GenScript Inc. (Piscataway, NJ, United States), Bmi-1 antibody from Santa Cruz Biotechnology (CA, United States). *Bgl*Ⅱ, *Hind*Ⅲ and T4DNA ligase were obtained from Promega. BGC823 human gastric cancer cell lines were received from the Chinese Academy of Science. RPMI 1640 and fetal bovine serum were supplied by Gibco BRL (Grand Island, NY, United States). Liposomes LipofectAmine™2000, G418, Trizol reagent and reverse transcription-polymerase chain reaction (RT-PCR) kit were purchased from Invitrogen (Carlsbad, CA, United States) and senescence β-galactosidase staining kit (Cell Signaling Technology, Beverly, MA, United States).

Methods

Selection of siRNA for Bmi-1 target sequence: The analysis and design of Promega siRNA target sequence scanned human *Bmi-1* gene sequence (NM_005180) was based on the design principle of siRNA target sequence. The 19bp siRNA target sequences, including 1104nt-1122nt (GGAGGAGGTGAATGATAAA) and 1356nt-1374nt (GAGAGATGGACTGACAAAT), were selected as the target sequence after the BLAST homology analysis. Two oligonucleotide hairpin DNA single strands were synthesized (1104F and 1104R, 1356F and 1356R), adding BamHI and XhoI endonuclease residues at the two ends. Two oligonucleotide hairpin DNA single strands demonstrated the following:
1104F: 5'-GATCCGGAGGAGGTGAATGATAAATTCAAGAGATTTATCATTCACCTCCTCCTTTTTTC-3',
1104R: 5'-TCGAGAAAAAAGGAGGAGGTGAATGATAAATCTCTTGAATTTATCATTCACCTCCTCCG-3';
1356F: 5'-GATCCGAGAGATGGACTGACAAATTTCAAGAGAATTTGTCAGTCCATCTCTCTTTTTTC-3',
1356R: 5'-TCGAGAAAAAAGAGAGATGGACTGACAAATTCTCTTGAAATTTGTCAGTCCATCTCTCG-3'.

Reconstruction of siRNA vectors: The single-stranded DNA oligonucleotide (1104F and 1104R, 1356F and 1356R) was converted into a double-stranded DNA (si1104 and si1356) by conventional annealing, and reconnected overnight at 4 ℃, utilizing 2 × reaction reconnected buffer (5 μL), linear pRNAT-U6.2 vector (1 μL), T4 ligase (1 μL) and annealing product (3 μL). The two recovered products were incubated at 16 ℃ for 16 h after addition of Solution I containing DNA ligase, and the resulting ligated products were used to transfect well-prepared competent *E. coli* DH5α. The whole transfection mix was plated onto a prewarmed LB-ampicillin (AMP) agar plate and then incubated at 37 ℃ for 12 h. Individual growing colonies were picked out and incubated at 37 ℃ for 12 h in LB broth containing AMP. Full length plasmid DNA was extracted from positive clones using a plasmid DNA extraction kit and then subject to testing for the presence of Bmi-1 with nuclease digestion using Bgl Ⅱ, Hind Ⅲ and T4DNA ligase.

Identification of recombinants: The recombinants were identified by PCR amplification, using primers PRNA-U6.2 FORWARD and PRNA-U6.2 REVERSE. PCR reaction was performed with 3 min of initial denaturation at 94 ℃, 35 cycles of 45 s denaturation at 94 ℃, 45 s annealing at 55 ℃, 45 s extension at 72 ℃, and finally 10 min extension at 68 ℃. RT-PCR amplification products were electrophoresed and inspected on a 1.1% agarose gel, and recovered and purified by using DNA Gel recovery kit.

Transfection by liposome-mediated siRNA: The transfection process was according to the Lipofectamine™ 2000 instructions: a cell suspension containing 4-8 × 10^5 cells was added to 500 μL of growth medium with serum but without antibiotics; 0.8-1.2 μg DNA was added to 50 μL of medium without serum; 2 μL of Lipofectamine™ 2000 was added to 50 μL OptiMEM® I medium and incubated for 5 min at room temperature; the DNA-Lipofectamine™ 2000 complexes were added and incubated for 4 h at 37 ℃ in a CO_2 incubator. Finally cells were assayed at 24-48 h post-transfection for the appropriate activity.

RT-PCR analysis: RT-PCR was carried out as described previously[8]. Cells were harvested and rinsed with phosphate-buffered saline (PBS) at corresponding time points and total RNA in the treated sections was extracted according to the total RNA extracting kit. A solution was added consisting of 10 mmol/L dNTP, 0.5 g/L oligo(dT), 40 U reverse transcriptase (m-mulv), 59 pH 8.3 RT buffer (250 mmol/L Tris-HCl, 250 mmol/L KCl, 20 mmol/L $MgCl_2$, 50 mmol DTT) and deionized

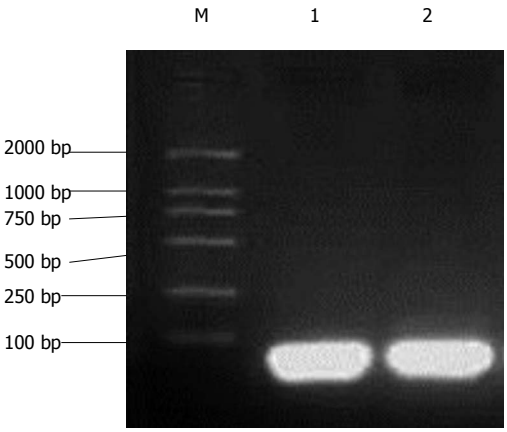

Figure 1 Annealing of siRNA hairpin DNA by electrophoresis. M: DNA marker; 1: Hairpin single-stranded DNA products for 1104F and 1104R; 2: Hairpin single-stranded DNA product for 1356F and1356R.

water. Total sample volume was 20 μL. Samples were incubated at 37 ℃ for 1 h and the reaction was stopped by heating at 70 ℃ for 10 min. Reverse transcriptase was used to synthesize the first-strand cDNA from an equal amount of the RNA sample following the manufacturer's instructions. About 35-45 cycles of PCR reaction were used to cover the linear range of the PCR amplification. The Bmi-1 specific primers (forward 5'GGAGACCAGCAAGTATTGTCC 3'; reverse 5'GACCATTCCTTCTCCAGGTAT 3') were used to amplify a 517 bp fragment of the *Bmi-1* coding region. β-actin was used as an internal control to amplify a 268 bp fragment. The band densities were scanned with a densitometer (Bio-Rad, United States). The relative amount of mRNA in each sample was calculated from the densitometry ratio of Bmi-1 OD value/β-actin OD value.

Western blotting analysis: Western blotting was conducted according to the manufacturer's instructions. The samples of each supernatant and the final pellets were heat-blocked for 5 min in a loading buffer (125 mmol/L Tris-HCl, 20%glycero1, 10%2-mercaptethanol, 4% SDS, 0.02% bromophenol blue, pH 6.8) and then subjected to electrophoresis on a 10%-20% Tris-glycine sulfate-polyacrylamide gel. The samples were then electronically transferred to a transfer membrane and blocked for 1 h in Tris-HCl buffered saline containing 5% skimmed milk and 0.1% Tween. Primary antibodies were incubated at 4-8 ℃ overnight in a TBS buffer containing 5% bovine albumin. The membrane was rinsed with TBS buffer containing 0.1% Tween 20, incubated with HRP-labeled second antibody for 2 h, and then stained with the detection reagents. Western blot analysis was performed as described previously to assess the protein expression level of Bmi-1 (1:200) and β-actin (1:100). Blots were developed with a SuperSignal ECL Western blotting Dura Substrate kit (Pierce Biotech, Rockford, IL, United States).

Senescence staining: Cell senescence β-galactosidase staining was carried out according to the manufacturer's instructions. Growth medium was removed from the cells and the plate rinsed once with PBS (2 mL for a 35 mm well), followed by addition of 1mL of 1x Fixative Solution to each 35 mm well. Cells were allowed to fix for 10-15 min at room temperature. The plate was rinsed twice with PBS (2 mL for a 35 mm well). After addition of 1 mL of β-galactosidase staining solution to each 35 mm well, the plate was incubated at 37 ℃ overnight in a dry incubator. While the β-galactosidase staining solution was still on the plate, the cells were checked under a microscope (× 200 total magnification) for the development of blue color. Five visual areas were randomly selected and photographed to record the percentage of the senescent cells.

Cell migration and invasion assay: Serum-free 1640 medium containing Matrigel was added to the filter membrane of the upper chamber to prepare a gel at 37 ℃ for 2 h. The 200 μL supernatant of serum-free NIH3T3 cells was utilized as chemokines in the lower chamber. After adding 400 μL of cells (1×10^9/L) to the upper chamber, they were cultured at 37 ℃ for 24 h. Five visual areas in the lower chamber were randomly selected and the percentage of senescent cells was recorded with hematoxylin-eosin staining. Each group had five parallel experiments.

Statistical analysis

Western blotting and RT-PCR results were analyzed with scanning densitometry (Bio-Rad). Quantitative data were documented as the mean ± SD. The significance of the differences was analyzed using SPSS 13.0 software (SPSS Inc., Chicago, IL, United States), with significance at $P < 0.05$.

RESULTS

Annealing of siRNA hairpin DNA

After annealing of hairpin single-stranded DNA for 1104 and 1356, the electrophoresis showed bright bands below 100 bp, consistent with the design (Figure 1).

Identification of Bmi-1 siRNA vectors

Two hairpin single-stranded DNA products (si1104 and si1356) were connected with pRNAT-U6.2 plasmid to transfect well-prepared competent *E. coli* DH5α. More than 10 transfected colonies grew on the Amp + LB culture plate. Ten transfected colonies were randomly selected. The DNA sequence of the inserted fragments was consistent with the designed positive recombinants (pRNAT-U6.2-si1104 and pRNAT-U6.2-si1356) (Figure 2).

Expression of Bmi-1 mRNA

The expression of Bmi-1 mRNA was inhibited in transfected BGC823 cells with pRNAT-U6.2-si1104 and pRNAT-U6.2-si1356, especially in pRNAT-U6.2-si1104 transfected BGC823 cells, while two control groups (empty vector and blank groups) had significantly higher levels of Bmi-1 mRNA ($P < 0.01$) (Figure 3A).

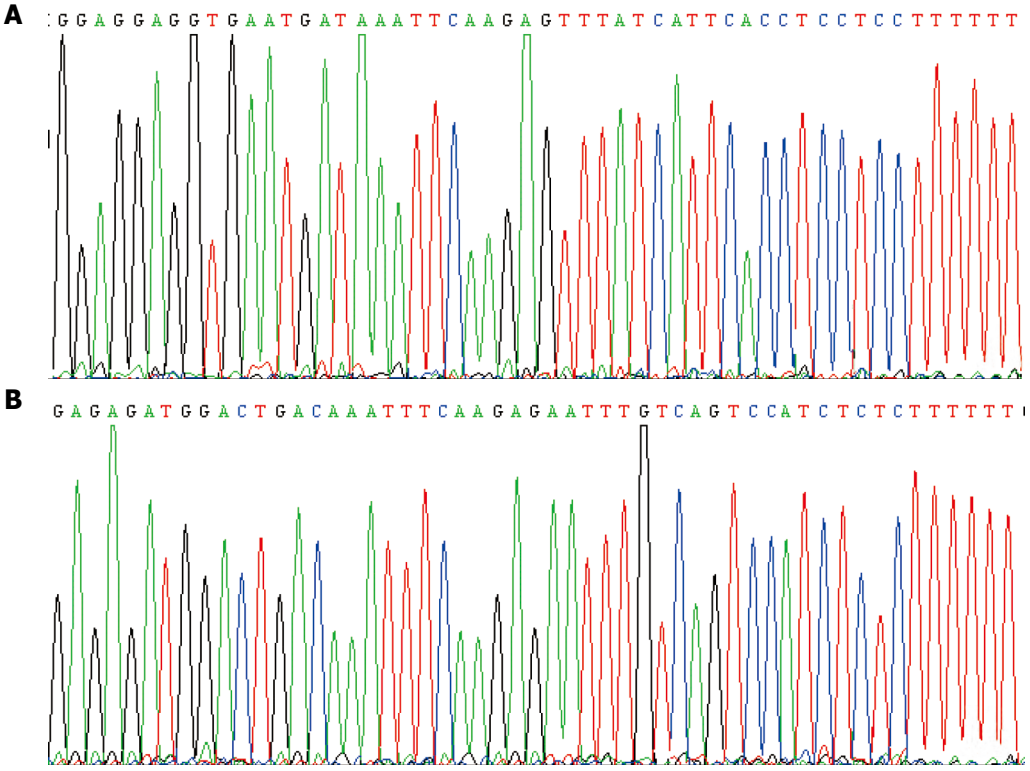

Figure 2 DNA sequence of the inserted fragment by transfected bacteria recombinant plasmid. A: The DNA sequence of the inserted fragment by recombinant plasmid pRNAT-U6.2-si1104. B: The DNA sequence of the inserted fragment by recombinant plasmid pRNAT-U6.2-si1356. The two DNA sequences of the inserted fragment by recombinant plasmids corresponded to the designed sequences.

Table 1 β-Gal activity assay and Boyden chamber assay to investigate the effects of Bmi-1 on cell senescence and metastasis (n = 5, mean ± SD)

Group	Transfected plasmids	β-Gal activity assay	Boyden chamber assay (cell number)
1	pRNAT-U6.2-si1104	28.3% ± 3.9%[b]	22.4 ± 4.2[b]
2	pRNAT-U6.2-si1356	25.9% ± 4.3%	33.6 ± 5.5[b]
3	pRNAT-U6.2	15.6% ± 2.7%	74.7 ± 9.3
4	Non-transfected	17.2% ± 3.1%	68.9 ± 10.1

Group 1: Transfected BGC823 cells with pRNAT-U6.2-si1104; Group 2: Transfected BGC823 cells with pRNAT-U6.2-si1356; Group 3: Transfected BGC823 cells with pRNAT-U6.2 (empty vector); Group 4: Non-transfected BGC823cells (blank). [b]$P < 0.01$ *vs* non-transfected and transfected BGC823 cells with empty vector pRNAT-U6.2.

Expression of Bmi-1 protein

There were high levels of Bmi-1 protein by Western blotting in non-transfected and transfected BGC823 cells with empty vector pRNAT-U6.2, compared with transfected BGC823 cells targeting Bmi-1 (pRNAT-U6.2-si1104 and pRNAT-U6.2 -si1356). while there was no Bmi-1 expression in the transfected BGC823 cells with pRNAT-U6.2-si1104 targeting Bmi-1 ($P < 0.01$) (Figure 3B).

Silencing Bmi-1 increased the senescent cell rate and reduced the metastasis of BGC823cells

The senescent rate of transfected BGC823 cells with pRNAT-U6.2-si1104 and pRNAT-U6.2-si1356 significantly increased compared with the non-transfected and transfected BGC823 cells with empty vector pRNAT-U6.2 ($P < 0.01$). The number of transfected BGC823 cells with pRNAT-U6.2-si1104 and pRNAT-U6.2-si1356 through the Matrigel significantly decreased, compared with the non-transfected and transfected BGC823 cells with empty vector pRNAT-U6.2 ($P < 0.01$) (Table 1).

DISCUSSION

This study aimed to investigate the impact of Bmi-1 on the senescence and metastasis of human gastric cancer cells, and our results indicate that inhibition of *Bmi-1* gene expression can enhance the senescence of human gastric cancer cells and limit the invasion and metastasis of human gastric cancer cells.

Gastric cancer, the most common gastrointestinal malignancy, is the fourth most commonly diagnosed malignancy and the second leading cause of cancer-related death in the world[9]. Gastric cancer is often either asymptomatic or has nonspecific symptoms in its early stages.

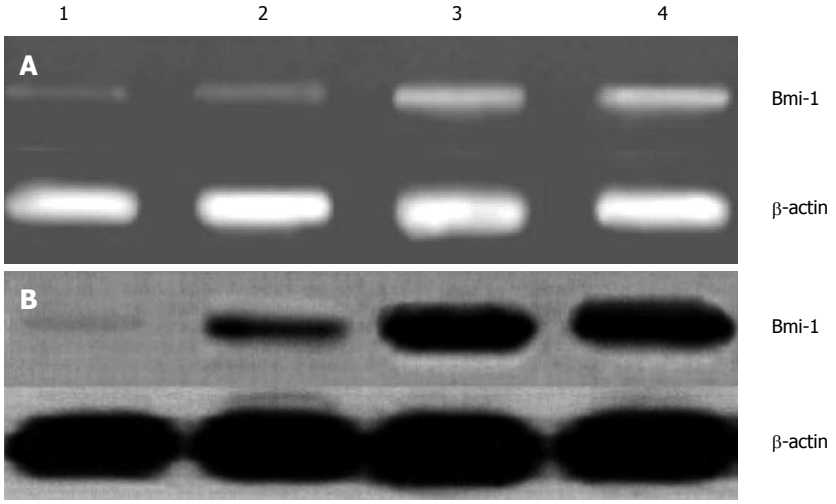

Figure 3 Levels of Bmi-1 mRNA and protein. A: The Bmi-1 mRNA level decreased in transfected BGC823 cells with pRNAT-U6.2-si1104 and pRNAT-U6.2-si1356, especially in pRNAT-U6.2-si1104 transfected BGC823 cells. B: The levels of Bmi-1 protein was higher in non-transfected and transfected BGC823 cells with empty vector pRNAT-U6.2, compared with transfected BGC823 cells targeting Bmi-1 (pRNAT-U6.2-si1104 and pRNAT-U6.2 -si1356). There was no expression in the transfected BGC823 cells with pRNAT-U6.2-si1104 targeting Bmi-1. 1: Transfected BGC823 cells with pRNAT-U6.2-si1104; 2: Transfected BGC823 cells with pRNAT-U6.2-si1356; 3: Transfected BGC823 cells with pRNAT-U6.2 (empty vector); 4: Non-transfected BGC823cells (blank).

Once symptoms become apparent, the cancer has often reached an advanced stage and may also have metastasized and spread to other parts of the body. Accordingly, gastric cancer has a relatively poor prognosis since invasion and metastasis are important prognostic factors[10,11]. Currently, there is evidence that the incidence of gastric cancer is related to multiple oncogenes, such as *C-myc*, *Ras*, *Hst* and *C-erbB-2*[12-14]. The *Bmi-1* gene, a polycomb gene (PcG), has been reported as an oncogene with high expression in cancers, and this may be related to high aggressiveness, such that overexpression of Bmi-1 is associated with poor prognosis[1,7]. Compelling research has supported that the expression of Bmi-1 decreases tumor cell senescence and proliferation, and increases tumor invasion and metastasis. The *Bmi-1* gene can be synergistic with C-myc to induce cell metastasis and tumor formation[3,15,16]. This study demonstrated that the inhibition of *Bmi-1* gene expression can increase the senescence of gastric cancer cells and slow down the invasion and metastasis of gastric cancer cells. It has provided further evidence of a role for Bmi-1 in the pathogenesis of gastric cancer.

The senescence β-galactosidase staining kit is designed to detect β-galactosidase activity at pH 6, a known characteristic of senescent cells not found in presenescent, quiescent or immortal cells[17,18]. Boyden chamber assays are used to measure cell invasion and various types of cell migration[19,20]. In this study, the incidence of senescent gastric cancer cells was most obvious when Bmi-1 expression was inhibited, according to β-galactosidase activity. Meanwhile, the number of gastric cancer cells through the Matrigel significantly decreased after inhibiting Bmi-1 expression in the Boyden chamber assay, indicating that the inhibition of Bmi-1 expression can limit the invasion and metastasis of gastric cancer cells. These results suggest that inhibition of *Bmi-1* gene expression can enhance cell senescence and reduce the capability for cell invasion and metastasis.

In conclusion, we documented in the present study that silencing Bmi-1 by RNA interference enhances the senescent cell rate and effectively reduces the metastasis of gastric cancer cells. Many studies have shown that Bmi-1 is essential in multiple pathways in the pathogenesis of gastric cancer. Other reports have suggested that Bmi-1 inhibitors have therapeutic potential for gastric cancer through various mechanisms. The current has provided additional support for the notion that Bmi-1 inhibitors might be developed as new agents for gastric cancer.

COMMENTS

Background

Bmi-1 (B lymphoma Mo-MLV insertion region 1 homolog) has been reported as an oncogene that plays an important role in several types of cancer. The amplification and overexpression of Bmi-1 contribute to the development of many tumors and cancers, such as skin, prostate, breast, ovarian, and colorectal, as well as hematological malignancies. Whether Bmi-1 influences cell senescence and metastasis of human gastric cancer remains unknown. The aim of this study was to evaluate the impact of Bmi-1 on cell senescence and metastasis of the human gastric cancer cell line BGC823.

Research frontiers

Bmi-1 is essential in multiple pathways in the pathogenesis of gastric cancer. The role of Bmi-1 on cell senescence and metastasis of human gastric cancer remains unclear.

Innovations and breakthroughs

The inhibition of *Bmi-1* gene expression can enhance the senescence of gastric cancer cells and limit the invasion and metastasis of gastric cancer cells.

Applications

Bmi-1 inhibitors have therapeutic potential for gastric cancer through various mechanisms. This research has provided additional support for the notion that Bmi-1 inhibitors might be developed as new agents for gastric cancer.

Peer review

This study demonstrated that the inhibition of *Bmi-1* gene expression can increase gastric cancer cell senescence and inhibit invasive behavior in a well-accepted Boyden chamber model. The present study focused on the role of Bmi in cell senescence and metastasis. It would help to understand the mechanism

of Bmi contribution to cancer progression. The data presented in this manuscript are quite good and very supportive of the hypothesis tested.

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META-ANALYSIS

Meta-analysis of Barrett's esophagus in China

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Abstract

AIM: To investigate the epidemiology and characteristics of Barrett's esophagus (BE) in China and compare with cases in the west.

METHODS: Studies were retrieved from the China National Knowledge Infrastructure and PubMed databases using the terms "Barrett" and "Barrett AND China", respectively, as well as published studies about BE in China from 2000 to 2011. The researchers reviewed the titles and abstracts of all search results to determine whether or not the literature was relevant to the current topic of this research. The references listed in the studies were also searched. Inclusion and exclusion criteria for the literature were appropriately established, and the data reported in the selected studies were analyzed. Finally, a meta-analysis was performed.

RESULTS: The current research included 3873 cases of BE from 69 studies. The endoscopic detection rate of BE in China was 1%. The ratio of male to female cases was 1.781 to 1, and the average age of BE patients was 49.07 ± 5.09 years. Island-type and short-segment BE were the most common endoscopic manifestations, accounting for 4.48% and 80.3%, respectively, of all cases studied. Cardiac-type BE was observed in 40.0% of the cases, representing the most common histological characteristic of the condition. Cancer incidence was 1.418 per 1000 person-years.

CONCLUSION: Average age of BE patients in China is lower than in Western countries. Endoscopic detection and cancer incidence were also lower in China.

Key words: Barrett's esophagus; Epidemiology; Cancer incidence; China; Meta-analysis

Core tip: Barrett's esophagus (BE) is a precursor of esophageal adenocarcinoma. Western countries have published more research on BE than China has. Thus, epidemiological knowledge of BE in China is inadequate. Diagnosis and treatment of BE in China is based on western criteria, therefore, diagnosis, monitoring, and treatment of BE require more data based on the unique characteristics of patients and clinics in China. The current research analyzed 69 clinical studies to obtain a comprehensive understanding of BE in China. Results provide important guidelines that can help improve the treatment and follow-up of BE patients in China.

Dong Y, Qi B, Feng XY, Jiang CM. Meta-analysis of Barrett's esophagus in China. *World J Gastroenterol* 2013; 19(46): 8770-8779 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i46/8770.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i46.8770

INTRODUCTION

Barrett's esophagus (BE) is a pathological phenomenon that occurs when the stratified squamous epithelium in the lower esophagus is replaced by a metaplastic simple columnar epithelium. In some cases, BE is accompanied by intestinal metaplasia, which is considered a precursor of esophageal adenocarcinoma[1]. Academics in Western countries have conducted more research on the subject than researchers in China. As such, despite the attention BE has drawn in recent years, epidemiological knowledge of BE in China is insufficient. The Digestive Disease Branch of the Chinese Medical Association drafted the Diagnosis and Treatment Consensus[2] of BE in 2005 and amended it in 2011, when a consensus amongst clinicians was finally achieved[1]. This consensus on BE, however, is based on western standards. Thus, the diagnosis, monitoring, and treatment of BE in China require more data based on Chinese clinics.

Although increasing numbers of researchers in China have focused on BE, the studies published thus far do not feature large samples or prospective designs. A systematic review[3] of the clinical characteristics of BE in China was published in 2008. However, in this review, studies that used metaplasia as a necessary standard were not excluded, which contradicts the consensus. In addition, the sample sizes of some included studies are rather small, with reports featuring only one or two cases. The present study aims to obtain a comprehensive understanding of the characteristics of BE in China by conducting a meta-analysis on BE in China and comparing findings with cases in Western countries. Results will help improve the treatment and follow-up of Chinese BE patients.

MATERIALS AND METHODS

Sources of literature and retrieval methods

Information from the China National Knowledge Infrastructure (CNKI) and PubMed databases were used. Clinical studies on BE published in Chinese between 2000 and 2011 were retrieved from the CNKI database, and those published in English were obtained from the PubMed database using the keywords "Barrett" and "Barrett AND China", respectively. The researchers reviewed the titles and abstracts of all search results to determine whether or not the study was relevant to the current topic. The references listed in the studies obtained were also reviewed to locate additional studies.

Inclusion criteria of studies

The selected studies met the following criteria: (1) all of the cases described were from China; (2) diagnosis of BE conformed to standards set by the Digestive Disease Branch of the Chinese Medical Association in 2011; (3) BE was diagnosed through endoscopy and pathology; and (4) the number of cases included in the sample was > 10.

Exclusion criteria of studies

Studies were excluded if they featured any of the following criteria: (1) only published as an abstract; (2) intestinal metaplasia was used as a necessary diagnostic criteria; (3) the clinical aspects of BE were insufficient; that is, the study lacked at least three of the following aspects: endoscopic detection rate, sex, age, endoscopic manifestations, or histological type; (4) only a short segment of the study focused on BE; or (5) duplicate publication.

Data extraction and statistical analysis

Four researchers independently extracted data from every study, and any ensuing disagreements were resolved through discussion. The following data were extracted: name of the first author; year of publication; region of study; total number of cases; male to female ratio of the patients; average age, endoscopic detection rate; proportion of each endoscopic and histological type; follow-up cases and follow-up duration; and occurrence of esophageal adenocarcinoma during follow-up.

Data were analyzed using SPSS version 17.0. Proportions were evaluated using standard formulas. A mean difference demonstrating a 95% confidence rate was used for continuous data. The total number of person-years during follow-up was calculated by multiplying the number of follow-up cases with the follow-up duration. Cancer incidence was calculated by dividing the number of occurrences of esophageal adenocarcinoma among the follow-up cases by the total number of person-years.

RESULTS

Sources of studies

A total of 1121 studies were found in the CNKI database, and 108 of these studies met the inclusion criteria. Among these studies, 42 were rejected on the basis of the exclusion criteria; seven[4-10] for using intestinal metaplasia as a necessary diagnostic standard; 28[11-38] for having insufficient data on the clinical aspects of BE; four[39-42] for providing only a small study on BE; and one each for inconsistent data[43], doubts about plagiarism[44] and duplicate publication[45]. A total of 65 studies were found in the PubMed database; five of which met the inclusion criteria. Among these studies, one[46] was excluded because of its duplicate publication in Chinese, and another[47] was excluded for its use of intestinal metaplasia as a necessary diagnostic standard. A total of 69[48-116] studies were included in the present research. The screening process is summarized using the flow diagram shown in Figure 1.

Characteristics of included studies

The 69 studies included in the present research were conducted in 25 provinces. The total number of samples in these studied was 12404, and the total number of cases was 3873 (Table 1).

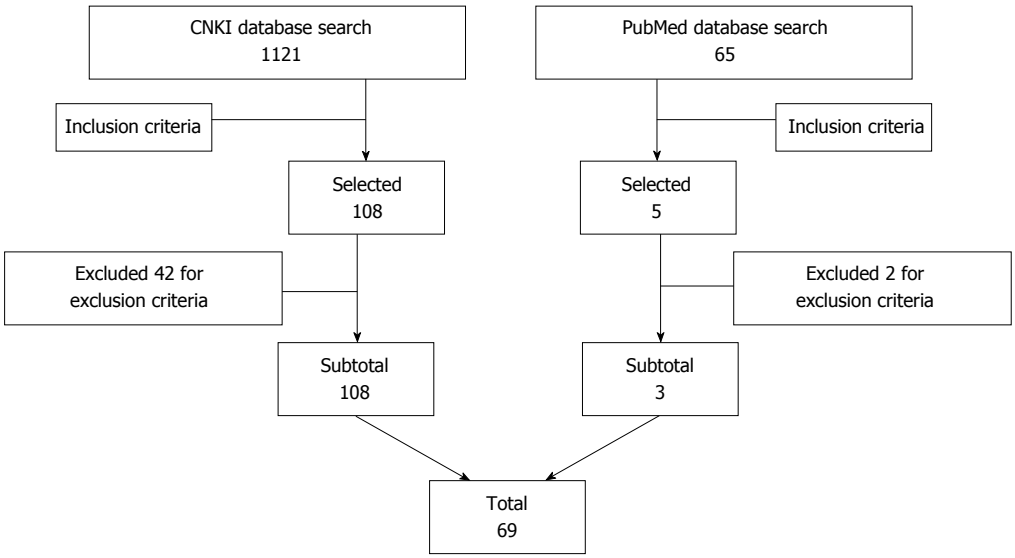

Figure 1 Flow diagram for the literature search. CNKI: China National Knowledge Infrastructure.

Endoscopic detection rate

A total of 15 studies reported the endoscopic detection rate of BE, which was obtained from all patients who had undergone endoscopy. However, the detection rate varied significantly, with rates ranging from 0.06% to 17.65%. The total endoscopic detection rate was 1.0% (95%CI: 0.1%-1.8%).

Sex

All studies reported the sex of BE patients (Table 1). One study[85] was not included in this analysis because the sum of male and female patients was inconsistent with the reported total number of cases. The remaining 68 studies showed a total of 3829 cases with 2452 male patients, accounting for 64.0% of the sample (95%CI: 61.1%-67.0%), and 1377 female patients, accounting for 36.0% of the sample (95%CI: 33.0%-38.9%). The male to female ratio was 1.781 (95%CI: 1.552-2.009).

Age

A total of 58 studies reported the age of the BE patients (Table 1), and the average age of the patients was 49.07 ± 5.09 years.

Endoscopic manifestations

The endoscopic patterns of BE in 49 studies could be categorized into several types based on Herlihy criteria[117]: island, tongue, circumferential, and mixed. On the basis of the columnar epithelial length reported in 29 studies, BE was divided into long-segment BE (LSBE) (*i.e.*, columnar epithelial metaplasia cells were involved in the entire circumference of the esophagus and the length of the segment was ⩾ 3 cm) and short-segment BE (SSBE) (*i.e.*, columnar epithelial metaplasia cells were not involved in the entire circumference of the esophagus or the whole circumference of esophagus was involved but the length of the segment was < 3 cm)[1] (Table 2).

Histological type

The histological type of BE was divided into the gastric-fundic, cardiac, and intestinal metaplasia types[1] (Table 3).

Cancer incidence of BE

Thirty-one studies reported follow-up information. The total number of follow-up cases was 1283, with a follow-up duration ranging from 3 mo to 3 years. The mean follow-up duration was 1.099 years. Three studies[80,91,95] focused only on the follow-up of atypical hyperplasia of BE; thus, these studies were not included in this analysis. The total number of person-years during follow-up was 1410. Among 1283 cases, only two developed esophageal adenocarcinoma during the follow-up period; these cases were reported in two studies[91,103]. Four cases were also detected with esophageal adenocarcinoma during follow-up, but the number of follow-up cases and follow-up times were not provided. Studies with these cases were excluded from this analysis. The cancer incidence of BE was 1.418 per 1000 person-years.

DISCUSSION

The present research included 69 studies. The total endoscopic detection rate of BE was 1.0%, consistent with the total BE morbidity rate in Asia (0.9%-1.2%) reported by Hou *et al*[118]. The endoscopic detection rate of BE in the reviewed studies ranged from 0.06% to 17.65%. Tseng *et al*[90] reported that the endoscopic detection rate of BE in Taiwan was 0.06%, which is much lower than the total detection rate observed in China. This variation may be attributed to their inclusion of upper gastrointestinal tract endoscopy in routine health maintenance programs, which can yield more reliable data on the prevalence of BE in local populations. The endoscopic detection rate of BE in most studies is based on patients who have undergone upper gastrointestinal tract endos-

Table 1 Characteristics of the selected studies

Ref.	Year of publication	Region (province)	Cases	Male	Female	Mean age (yr)
Chen *et al*[48]	2011	Henan	150	60	90	45.42
Chen *et al*[49]	2011	Hubei	52	31	21	49
Guo *et al*[50]	2011	Hebei	42	27	15	48
Han *et al*[51]	2011	Jilin	30	21	9	45
Hao *et al*[52]	2011	Guangdong	76	58	18	50.6
Lin *et al*[53]	2011	Zhejiang	41	28	13	58.9
Lv *et al*[54]	2011	Zhejiang	108	80	28	61
Su *et al*[55]	2011	Hubei	23	13	10	40.3
Wang *et al*[56]	2011	Fujian	113	67	46	54.5
Zou *et al*[57]	2010	Guangxi	23	16	7	50.3
Xia *et al*[58]	2010	Hubei	56	34	22	45.85
Wu *et al*[59]	2010	Shanghai	84	50	34	36.3
Liu *et al*[60]	2010	Chongqing	62	35	27	51
Li *et al*[61]	2010	Anhui	32	18	14	48.6
Li *et al*[62]	2010	Guangdong	45	18	27	43
Hao *et al*[63]	2010	Henan	144	98	46	NA1
Yao *et al*[64]	2010	Sichuan	21	15	6	40.1
Tang *et al*[65]	2010	Jiangxi	63	43	20	45
Shi *et al*[66]	2010	Fujian	57	36	21	53
Jia *et al*[67]	2010	Shanxi	26	21	5	49
Gao *et al*[68]	2010	Hubei	32	21	11	NA
Tian *et al*[69]	2009	Shandong	59	43	16	49.14
Li *et al*[70]	2009	Guangxi	38	27	11	47.56
Dai *et al*[71]	2009	Hunan	23	18	5	50.3
Bai *et al*[72]	2009	Chongqing	67	41	26	50.7
Yang *et al*[73]	2009	Shaanxi	87	58	29	53.3
Yang *et al*[74]	2009	Ningxia	51	29	22	49.14
Tan *et al*[75]	2009	Liaoning	48	31	17	58.4
Qiu *et al*[76]	2009	Fujian	404	238	166	44.2
Lu *et al*[77]	2009	Jiangsu	12	9	3	NA
Liu *et al*[78]	2009	Liaoning	23	18	5	49
Gao *et al*[79]	2009	Liaoning	42	25	17	NA
Peng *et al*[80]	2009	Guangdong	27	14	13	48.18
Wu *et al*[81]	2008	Henan	25	16	9	48.3
Wang *et al*[82]	2008	Henan	12	10	2	49.5
Wang *et al*[83]	2008	Ningxia	109	64	45	50.11
Lu *et al*[84]	2008	Guangxi	32	22	10	52.5
Gao *et al*[85]	2008	Shandong	44	22	20	50
Zhang *et al*[86]	2008	Jiangxi	84	51	33	46
Yang *et al*[87]	2008	Hebei	74	40	34	52.6
Jian *et al*[88]	2008	Yunnan	68	51	17	52
Ji *et al*[89]	2008	Jiangsu	51	38	13	52.5
Tseng *et al*[90]	2008	Taiwan	12	9	3	61.6
Zhang *et al*[91]	2007	Shandong	30	24	6	52
Meng *et al*[92]	2007	Liaoning	21	13	8	54.6
Duan *et al*[93]	2007	Henan	54	38	16	51.6
Zhou *et al*[94]	2007	Zhejiang	13	7	6	NA
Wang *et al*[95]	2007	Hubei	88	61	27	47.46
Li *et al*[96]	2007	Fujian	75	45	30	45.42
Jin *et al*[97]	2007	Zhejiang	37	22	15	53
Zhou *et al*[98]	2006	Hubei	128	93	35	NA
Yang *et al*[99]	2006	Shaanxi	86	58	28	46
Wu *et al*[100]	2006	Fujian	13	10	3	48
Wang *et al*[101]	2006	Shaanxi	73	29	44	45.6
Suo *et al*[102]	2006	Fujian	37	24	13	50
Li *et al*[103]	2006	Liaoning	54	35	19	49
Li *et al*[104]	2006	Tianjin	37	25	12	58.3
Dou *et al*[105]	2006	Guizhou	89	57	32	46.3
Wang *et al*[106]	2006	Hubei	33	22	11	48
Shu *et al*[107]	2006	Hubei	13	12	1	NA
Zheng *et al*[108]	2005	Hubei	45	31	14	NA
Liang *et al*[109]	2005	Xinjiang	20	14	6	NA
Zhang *et al*[110]	2004	Shaanxi	69	54	15	56.2
Zhao *et al*[111]	2003	Shandong	55	38	17	46.8
Dong *et al*[112]	2003	Zhejiang	32	23	9	48.8
Zhang *et al*[113]	2001	Anhui	14	11	3	NA
Wang *et al*[114]	2001	Guangdong	21	16	5	67.3

Zhao *et al*[115]	2000	Shandong	35	26	9	NA
Yang *et al*[116]	2000	Beijing	29	22	7	50

NA: Not applicable (data were either unavailable or not reported).

Table 2 Endoscopic manifestations of Barrett's esophagus

Type	Proportion	95%CI
Island	0.448	0.375-0.521
Tongue	0.262	0.204-0.320
Circumferential	0.247	0.190-0.303
Mixed	0.043	-0.006-0.093
SSBE	0.803	0.771-0.835
LSBE	0.197	0.165-0.229

The island type of Barrett's esophagus (BE) accounted for 44.8% of all cases, the tongue type for 26.2%, the circumferential type for 24.7%, and the mixed type for 4.3%. Short-segment BE and long-segment BE accounted for 80.3% and 19.7% of the cases, respectively.

Table 3 Histological type of Barrett's esophagus

Type	Proportion	95%CI
Cardiac	0.400	0.310-0.491
Gastric-fundic	0.325	0.227-0.422
Intestinal Metaplasia	0.272	0.226-0.318
Mixed type	0.003	-0.002-0.008

The cardiac type accounted for 40.0% of the cases, the gastric-fundic type for 32.5%, the intestinal metaplasia type for 27.2%, and the mixed type for 0.3%.

copy in a local hospital. These patients mainly suffer from several gastrointestinal symptoms such as regurgitation, heartburn, epigastric discomfort, nausea, vomiting, and eructation. Reports of such symptoms increase the detection rate of BE, so this result cannot represent the prevalence of BE in the general population.

In this research, the total endoscopic detection rate was lower than that reported in a meta-analysis in 2008[3] (2.39%), likely because of the increasing number of patients accepting endoscopy in recent years as a means of diagnosing and treating upper gastrointestinal tract diseases. Some patients opt to undergo endoscopic examination when experiencing upper gastrointestinal tract symptoms, while others choose endoscopy for routine health maintenance. Thus, the data do not provide sufficient evidence to conclude that the incidence of BE has declined in China.

The detection rate of BE in Western countries is 3%-8%[119], which is higher than that in China. Variations observed may be due to the following reasons: (1) variations in genetic and environmental factors; (2) western lifestyle and diet-related factors, such as visceral obesity, high-fat diet, and tobacco and alcohol consumption, which are risk factors for BE[118,120-133]; and (3) delayed recognition of BE in China, considering that current diagnostic standards are based on western practices and some Chinese doctors experience difficulties when diagnosing patients with BE.

In this research, the number of male BE patients was higher than that of female patients, which is similar to western reports[133]. The average age of onset of BE in this study was 49.07 years, whereas the average age in Western countries is 60 years[134,135]. Variations observed may be attributed to differences in the Chinese and western lifestyles.

The main types of endoscopic manifestations of BE include the island type and SSBE, which is similar to western reports[136]. Experts in the United States believe that LSBE and SSBE represent two different pathological changes and that the former is related to severe gastroesophageal reflux disease, which is common in older people. Although LSBE tends to increase the risk of cancer, no evidence today correlates the length of BE and cancer risk[137].

The number of cases of cardiac type BE was higher than that of other histological types in this research. Jankowski *et al*[138] reported that intestinal metaplasia progresses to cancer, and the Diagnosis and Treatment Consensus of BE of China (2011) regards intestinal metaplasia as a precursor of esophageal adenocarcinoma. Unfortunately, the reviewed studies present limited clinical and pathological data on intestinal metaplasia related to BE, considering Chinese researchers' lack of knowledge on the topic. Some researchers believe that cancer formation is related to atypia of the epithelial instead of columnar epithelial metaplasia[139].

An individual with BE is estimated to be at 25-30 times greater risk of developing esophageal adenocarcinoma[140-143] than the general population. Cancer incidence was found to be 1.418 per 1000 person-years in the present study, which is lower than that in England (7.0 per 1000 person-years), United States (6.4 per 1000 person-years), and other European countries (5.6 per 1000 person-years)[144]. Thus, the cancer incidence of BE in China is lower than that in Western countries. The spectrum of BE characteristics differs significantly between the two regions.

COMMENTS

Background

Barrett's esophagus (BE) is a precursor of esophageal adenocarcinoma. The Diagnosis and Treatment Consensus of BE in China is based on Western criteria. Epidemiological knowledge of BE in China is inadequate.

Research frontiers

A large number of clinical studies on BE have been conducted in China but they do not feature large sample sizes or prospective designs. A systematic review of the clinical characteristics of BE in China was published in 2008. However, in this review, studies that used metaplasia as a necessary standard were not excluded, which contrasts the consensus.

Innovations and breakthroughs

The Digestive Disease Branch of the Chinese Medical Association drafted the

Diagnosis and Treatment Consensus of BE in 2005 and amended it in 2011, when a consensus amongst clinicians was achieved. Based on this consensus, the present research analyzed existing clinical studies with the aim of obtaining a comprehensive understanding of the characteristics of BE in China.

Applications

This research analyzed existing clinical studies with the aim of understanding the characteristics of BE in China. The results obtained will help improve the treatment and follow-up protocols of BE patients in China.

Terminology

BE is a pathological phenomenon that occurs when the stratified squamous epithelium in the lower esophagus is replaced by a metaplastic simple columnar epithelium. In some cases, BE is accompanied by intestinal metaplasia, which is considered a precursor of esophageal adenocarcinoma.

Peer review

This research has a high degree of significance. The meta-analysis presented here reviews the characteristics of the BE cases in China, including patient demographics, endoscopic and histological features, and risks for developing adenocarcinoma. The inclusion and exclusion criteria are appropriate, and the information obtained from selected reports is sufficiently analyzed.

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META-ANALYSIS

Smoking, alcohol consumption, and the risk of extrahepatic cholangiocarcinoma: A meta-analysis

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Abstract

AIM: To assess the association between smoking and alcohol consumption and extrahepatic cholangiocarcinoma (ECC) through a meta-analysis of clinical observational studies.

METHODS: A literature search was conducted using Embase and MEDLINE databases from inception to 31 May 2013 without language limitations, and by manually searching the references of retrieved articles. Case-control and cohort studies that investigated the association between smoking or alcohol consumption and ECC were included. The quality of these studies was assessed using the Newcastle-Ottawa quality assessment scale. Summary relative risks and corresponding 95%CI were calculated using a random-effects model. Publication bias was assessed by Begg's funnel plot and Egger's test.

RESULTS: A total of 12 eligible articles (11 case-control studies and one cohort study) were included in this meta-analysis. Eleven studies reported the association between smoking and ECC. Pooled analysis indicated that smokers had an increased risk of ECC development as compared with non-smokers (summary RR = 1.23; 95%CI: 1.01-1.50). This correlation was present in population-based studies (n = 5; summary RR = 1.47; 95%CI: 1.06-2.05) but not in hospital-based studies (n = 6; summary RR = 1.10; 95%CI: 0.88-1.37) and in non-Asian regions (n = 7; summary RR = 1.39; 95%CI: 1.03-1.87) but not in Asia (n = 4; summary RR = 1.08; 95%CI: 0.85-1.38). Seven studies reported an association between consuming alcohol and ECC. Pooled analysis indicated that alcohol drinkers had a similar risk of ECC development as did individuals who did not drink alcohol (summary RR = 1.09; 95%CI: 0.87-1.37). There was moderate heterogeneity among the studies and no evidence of publication bias.

CONCLUSION: Smoking is associated with an increased risk of ECC, but alcohol consumption is not. Further population-based studies, particularly cohort studies, are warranted to enable definitive conclusions.

Key words: Extrahepatic cholangiocarcinoma; Smoking; Alcohol consumption; Meta-analysis; Relative risk

Core tip: Little is known about the etiology of extrahepatic cholangiocarcinoma (ECC) because of its rarity and high fatality. Smoking and alcohol consumption are potential risk factors for ECC development. However, reported relations between these two risk factors and ECC are conflicting. Our meta-analysis identified a positive association between smoking and the risk of ECC. The association between alcohol consumption and the

risk of ECC was positive but not significant. Further investigations are required.

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INTRODUCTION

An extrahepatic cholangiocarcinoma (ECC) is a malignant tumor that arises from cholangiocytes and involves the biliary tree within the hepatoduodenal ligament[1]. It is a relatively rare but often lethal neoplasm that accounts for about 80% of cholangiocarcinomas in the Western world[2]. The prognosis of ECC is poor, and the 5-year survival rate for patients with ECC after resection is as low as 20%-40%[3]. Hilar cholangiocarcinoma is typically classified as extrahepatic[4]. Although the incidence of ECC seems to be constant (annual percent changes = 1%)[1,5,6], it varies across regions, with the highest incidence in Southeast Asia and the lowest in Australia[7,8]. Such geographic variation may be associated with different genetic and environmental factors, including dietary patterns and lifestyle effects.

Although little is known about the etiology of ECC, several risk factors have been proposed to be involved in the development of this disease[9-11]. Epidemiological studies have found that a history of cholecystectomy, cholecystitis, parasitic infection, or primary sclerosing cholangitis is a risk factor for ECC[11-13]. There are other potential factors, such as hepatitis virus infection, obesity, diabetes, and host genetic polymorphisms, but these are less well established[4]. The low incidence of ECC precludes carrying out well-designed, single-center, case-control or prospective cohort studies with sufficient size and statistical power to determine the potential risk factors.

Smoking is associated with the risk of nonpulmonary cancer at many sites, including the liver and pancreas[14,15], and consuming alcohol is related to cancer of the upper digestive tract[16]. The metabolites of smoking and alcohol have carcinogenic properties[17]. However, the reported correlations between these two risk factors and ECC are inconsistent[10,11,18-27]. The lack of consistency across studies may be due to the small number of cases, differences in the study populations, differences in methodological designs or exposure definitions, or a shortage of data concerning confounding factors.

To provide a quantitative assessment of the correlations between these two factors and the risk of ECC, we performed a meta-analysis of published studies following the meta-analysis of observational studies in epidemiology guidelines[28].

MATERIALS AND METHODS

Data sources and searches

Two investigators (Ye XH and Huai JP) independently performed a computerized search of MEDLINE (from 1 January 1966 to 31 May 2013) and Embase (from 1 January 1974 to 31 May 2013) databases to identify potentially relevant articles. Searches were performed using the following text words and/or Medical Subject Headings: "tobacco", "smoking", "alcohol", "beverages", "ethanol", "cholangiocarcinoma", "extrahepatic", "bile duct cancer", and "epidemiologic studies"; the search results were restricted to studies performed after 1990 to avoid any possible inconsistencies in the diagnostic criteria used. The bibliographies of all relevant articles were reviewed manually to identify additional relevant articles. No language restrictions were imposed.

Study selection

Studies were included if they fulfilled the following criteria: (1) case-control or cohort design and published in manuscript form; (2) smoking or alcohol consumption included as an exposure of interest; (3) ECC included as an outcome of interest; and (4) RR in cohort studies or OR in case-control studies and their 95%CI (or sufficient data to calculate them) reported. If data on the same population were reported in multiple papers, the most informative report was selected. Studies were excluded if the data were not specified for ECC, or if they reported data for another type of cancer. Articles or reports that were not peer reviewed were not included.

Data extraction

Two investigators (Ye XH and Huai JP) independently extracted the following data from all included studies: first author's last name, publication year, geographic location of the study population, study design, methods used to determine risk factors and ECC, sample size (cases and controls or cohort size), variables adjusted for in the analysis, and RR estimates with corresponding 95%CI. From each study, the risk estimates that indicated the greatest degree of control for potential confounders were extracted, and discrepancies were resolved by consensus.

Assessment of study quality

The quality of the included studies was assessed using the Newcastle-Ottawa scale[29]. The scale consists of nine items that cover three dimensions: (1) patient selection (four items); (2) comparability of the two study arms (two items); and (3) assessment of outcome (three items). A point is awarded for each item that is satisfied by the study. The total score therefore ranges from zero to nine, with higher scores indicating higher quality. Studies that scored seven or more points were considered to be of high quality. The Newcastle-Ottawa scale score was assessed independently by both of the reviewers. Discrepancies in the score were resolved through discussion between the reviewers.

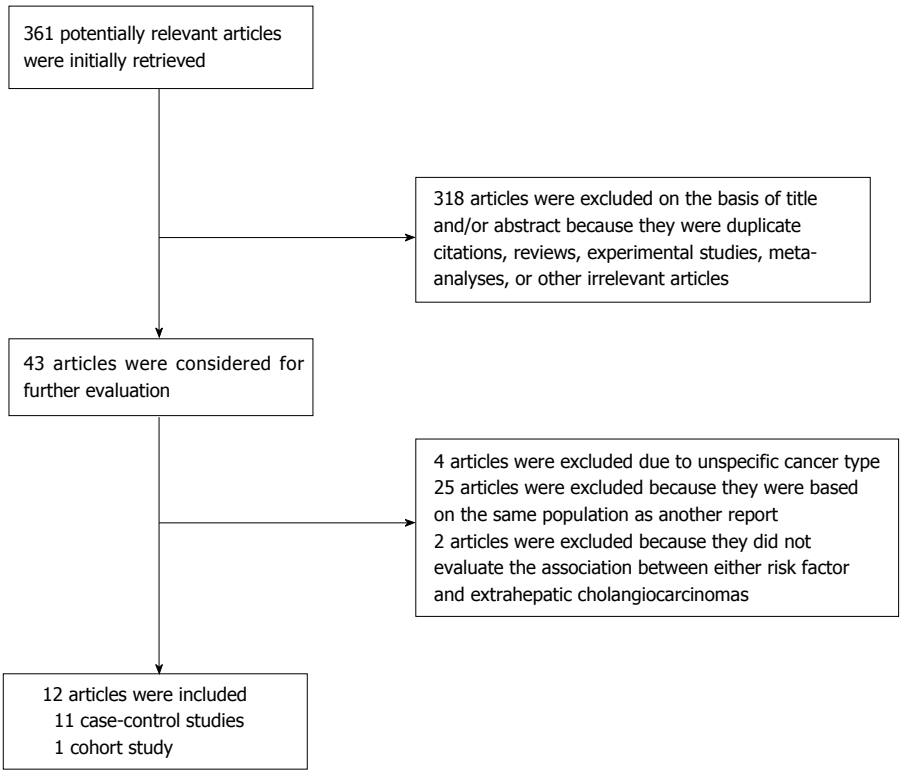

Figure 1 Flow chart of the study selection.

Statistical analysis

Different measures of RR were included in this meta-analysis: case-control studies (odds ratio) and cohort studies (rate ratio, hazard ratio). In practice, these measures of effect yielded similar estimates of RR because of the low absolute risk of ECC.

Summary RR estimates with their corresponding 95%CI were calculated with a random-effects model using the methods of DerSimonian and Laird, which consider both within- and between-study variations[30]. Most of the included studies reported the RR of ECC for smokers *vs* nonsmokers and alcohol drinkers *vs* non-alcohol drinkers. If studies reported separate RRs for males and females or for different levels of alcohol consumption, we calculated the pooled RR and its corresponding 95%CI. We conducted further analyses stratified by study design, geographic region, and adjustment for cholelithiasis.

Heterogeneity was evaluated using the Q-statistic and quantified by I^2[31]. For the Q test, a P value of > 0.10 was considered to indicate no statistically significant heterogeneity. I^2 is the proportion of total variation contributed by between-study variation. In addition, a sensitivity analysis was carried out to estimate the effects of each included study on the overall pooled RR. Publication bias was assessed using Begg's funnel plot and Egger's test[32,33]. All statistical analyses were carried out using STATA software (*vs* 12.0; STATA, College Station, Texas, United States). A two-tailed P value of < 0.05 was considered to be significant.

RESULTS

Search results and study characteristics

Twelve articles (11 case-control studies and 1 cohort study) were included in this meta-analysis (Figure 1). Eleven studies reported the association between smoking and ECC, and seven studies reported the association between alcohol consumption and ECC. Briefly, our initial search identified 361 articles, and 318 were excluded by examining the titles and abstracts. Reasons for the exclusion included duplicate citations, reviews, experimental studies, meta-analyses and other irrelevant articles. Forty-three full-text articles were considered for detailed evaluation. One additional relevant study was identified by manually reviewing the references of all 43 articles. Thirty-one of these 43 articles were subsequently excluded from the meta-analysis: 4 did not specify the cancer type, 25 were duplicate reports based on the same population, and 2 did not evaluate the association between each risk factor and ECC. The remaining 12 studies were published between 1993 and 2013 and included a total of 1834 incident cases (Table 1). The studies were carried out in Asia ($n = 4$), North America ($n = 6$), and Europe ($n = 2$; Table 1). Ten of the 12 studies were of high quality (Newcastle-Ottawa scale score ≥ 7; Table 2).

Control subjects in the 11 case-control studies were recruited from a population-based[11,18,19,21,26] or hospital-based setting[10,20,23-25,27] (Table 1). Most studies used a questionnaire or hospital records to evaluate smoking or alcohol consumption status (Table 1). ECC was diagnosed on the basis of histological and imaging methods in 10 studies and according to diagnostic codes in 1 study[11]; the method of diagnosis was not reported in 1 study[18] (Table 1). Adjustments were made for potential confounders of one or more factors in nine of 12 studies (Table 1).

One study[18] reported an increased risk of ECC in

Table 1 Characteristics of the 12 studies that reported an association between smoking or alcohol consumption and the risk of extrahepatic cholangiocarcinoma

Author and year	Country	Design	Source	Number of cases	Number of controls	Risk factor assessment	ECC ascertainment	Smoking RR (95% CI)	Alcohol RR (95% CI)	Adjustments
Ghadirian *et al*[18] 1993	Canada	Case-control	Population	24	239	Questionnaire	NA	2.820 (1.010-7.860)	-	Age, sex, other smoking habits, alcohol consumption, schooling, respondent status
Chow *et al*[19] 1994	United States	Case-control	Population	64	255	Questionnaire	Pathological	1.630 (0.900-2.970)[1]	0.600 (0.290-1.220)[1]	Age, ethnic origin, smoking status (adjusted for alcohol consumption)
Khan *et al*[20] 1999	United States	Case-control	Hospital	31	138	Medical records	Pathological	0.630 (0.210-1.880)	-	Age, female gender, ethnicity, cholelithiasis, socioeconomic status
Zhang *et al*[21] 2004	China	Case-control	Population	99	373	Questionnaire	Cancer registry	1.490 (0.870-2.560)[2]	1.290 (0.780-2.170)[2]	Age, total energy, cholelithiasis, hypertension, history of salty food intake, smoking status (adjusted for alcohol consumption)
Welzel *et al*[11] 2007	United States	Case-control	Population	549	102782	Medical records	Cancer registry	1.700 (1.000-3.000)	-	Age, sex, race, geographic location, state buy-in status
Shaib *et al*[10] 2007	United States	Case-control	Hospital	163	236	Medical records	Pathological + imaging	1.300 (0.800-1.900)[3]	1.290 (0.190-8.910)[1]	Race, age, gender, HBV, HCV markers (adjusted for alcohol consumption)
El-Serag *et al*[22] 2009	United States	Cohort	-	-	-	Registry	Cancer registry	-	1.060 (0.600-1.870)	Age, sex, baseline visit date, type of visit, a preceding visit
Tao *et al*[23] 2010	China	Case-control	Hospital	129	380	Medical records	Pathological + imaging	0.900 (0.500-1.300)[3]	1.200 (0.800-1.900)[3]	-
Cai *et al*[24] 2011[4]	China	Case-control	Hospital	313	608	Medical records	Pathological	0.900 (0.640-1.248)[3]	-	-
Onal *et al*[25] 2012	Turkey	Case-control	Hospital	89	48	Questionnaire	Pathological + imaging	1.900 (0.900-4.200)[3]	4.010 (0.480-33.62)[3]	-
Brandi *et al*[26] 2013	Italy	Case-control	Population	59	212	Questionnaire	Pathological	0.780 (0.400-1.500)	-	Age, sex, region of residence
Zhou *et al*[27] 2013	China	Case-control	Hospital	239	478	Medical records	Pathological + imaging	1.301 (0.863-1.962)	1.053 (0.670-1.655)	Age, sex, cirrhosis, cholelithiasis, cholecystectomy, DM, family history of other cancer

[1]The summary RRs and 95%CI were derived by pooling the relative risks for each sex; [2]Only males were included in the summary relative risks because of the rarity of smoking in females; [3]Univariate OR was calculated because adjusted ORs were not available; [4]Only hilar cholangiocarcinomas were included; ECC: Extrahepatic cholangiocarcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus; DM: Diabetes mellitus; NA: Not available.

smokers as compared with non-smokers, whereas 10 studies reported a similar risk of ECC in smokers and non-smokers (Table 1). All seven studies reported a similar risk of ECC in alcohol drinkers and non-alcohol drinkers (Table 1). One study[10] stratified alcohol consumption as moderate/heavy and found an increased risk of ECC in heavy drinkers as compared with non-drinkers (RR = 3.6; 95%CI: 1.5-9.4).

Only one study had a prospective cohort design and evaluated the association between alcohol consumption and the risk of ECC[22]. A total of 75 incidence cases of ECC were reported. An increased risk of ECC was observed in alcohol drinkers as compared with non-alcohol drinkers, but this was not significant.

Smoking and risk of ECC

Eleven case-control studies were identified that reported the association between smoking and the risk of ECC[10,11,18-21,23-27]. The summary RR for ECC was 1.23 (95%CI: 1.01-1.50) in a random-effects model for smokers vs non-smokers (Figure 2A). There was moderate heterogeneity among studies (Q = 14.69, P = 0.144 for heterogeneity, I^2 = 31.9%).

Subgroup meta-analyses were conducted according to geographical region, study design and confounders. The summary RR for ECC was significant for studies conducted outside of Asia[10,11,18-20,25,26] (n = 7; summary RR = 1.39; 95%CI: 1.03-1.87; P = 0.218 for heterogeneity, I^2 = 27.5%) but not for studies conducted in Asia (n = 4; summary RR = 1.08; 95% CI, 0.85-1.38; P = 0.278 for heterogeneity, I^2 = 22.1%; Table 3). The summary RR for ECC was significant for population-based case-control studies[11,18,19,21,26] (n = 5; summary RR = 1.47; 95%CI: 1.06-2.05; P = 0.243 for heterogeneity, I^2 = 26.8%) but not for hospital-based case-control studies (n = 6; summary RR =

Table 2 Quality of the studies used in this analysis

Author and year	Quality indicators of Newcastle-Ottawa quality assessment scale									Score
	Selection				Comparability		Exposure/outcome			
	Ⅰa	Ⅰb	Ⅰc	Ⅰd	Ⅱa	Ⅱb	Ⅲa	Ⅲb	Ⅲc	
Ghadirian *et al*[18] 1993	No	Yes	Yes	Yes	Yes	Yes	Yes	No	No	6
Chow *et al*[19] 1994	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	7
Khan *et al*[20] 1999	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	7
Zhang *et al*[21] 2004	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	7
Welzel *et al*[11] 2007	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	7
Shaib *et al*[10] 2007	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	7
El-Serag *et al*[22] 2009	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	8
Tao *et al*[23] 2010	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	7
Cai *et al*[24] 2011	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	No	7
Onal *et al*[25] 2012	Yes	Yes	No	Yes	Yes	No	Yes	Yes	No	7
Brandi *et al*[26] 2013	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No	6
Zhou *et al*[27] 2013	Yes	Yes	No	Yes	Yes	No	Yes	Yes	No	7

For case-control studies; Ⅰa: Indicates cases with independent validation; Ⅰb: Indicates consecutive or representative cases; Ⅰc: Indicates community controls; Ⅰd: Indicates controls with no history of ECC; Ⅱa: Indicates that study controls were comparable for age and sex; Ⅱb: Indicates that study controls were comparable on all additional factor(s) reported; Ⅲa: Indicates that the same method of ascertainment was used for cases and controls; Ⅲb: Indicates that assessment of exposure was from a secure record; Ⅲc: Indicates that the non-response rate was similar in both groups. For cohort studies; Ⅰa: indicates that the exposed cohort was representative of the population; Ⅰb: Indicates that the non-exposed cohort was drawn from the same population; Ⅰc: Indicates that the exposure ascertainment was from secure records or a structured interview; Ⅰd: Indicates that ECC was not present at start of study; Ⅱa: Indicates that the cohorts were comparable for age and sex; Ⅱb: Indicates that the cohorts were comparable on all additional factor(s) reported; Ⅲa: Indicates that ECC was assessed from a secure record; Ⅲb: Indicates that follow-up was long enough for ECC to occur; Ⅲc: Indicates that follow-up was complete. ECC: Extrahepatic cholangiocarcinoma.

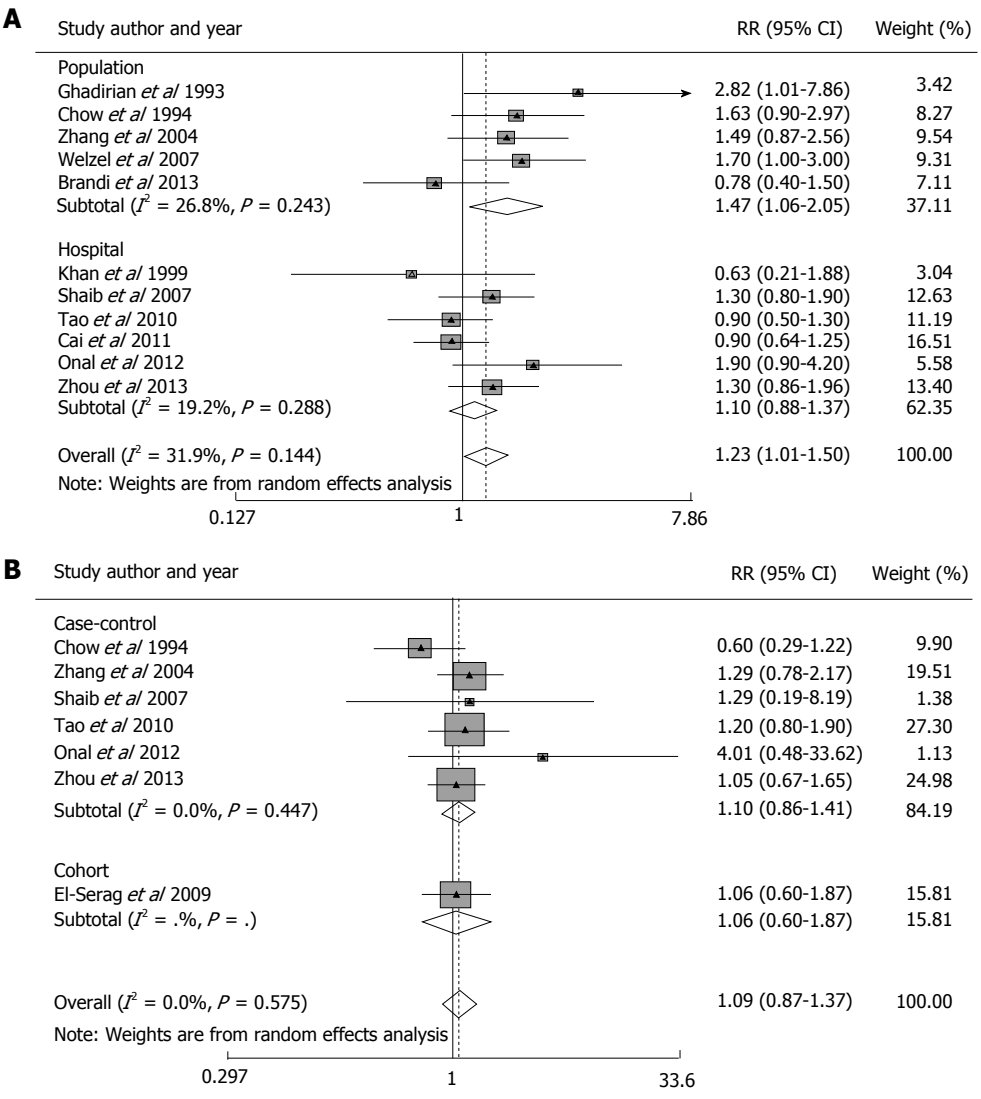

Figure 2 Relative risk of extrahepatic cholangiocarcinoma. A: Smokers as compared with non-smokers in population- and hospital-based case-control studies; B: Alcohol drinkers as compared with non-alcohol drinkers in case-control and cohort studies.

Table 3 Subgroup analyses of the association between smoking and extrahepatic cholangiocarcinoma and alcohol consumption and extrahepatic cholangiocarcinoma

	No. of studies	RR (95%CI)	Tests for heterogeneity		
			Q	*P*	I^2
Smoking					
Geographical region					
Non-Asia	7	1.39 (1.03-1.87)	8.280	0.218	27.5%
Asia	4	1.08 (0.85-1.38)	3.850	0.278	22.1%
Study design					
Population-based	5	1.47 (1.06-2.05)	5.470	0.243	26.8%
Hospital-based	6	1.10 (0.88-1.37)	6.190	0.288	19.2%
Adjustment for cholelithiasis	3	1.28 (0.94-1.76)	1.917	0.383	0.0%
Alcohol drinking					
Geographical region					
Non-Asia	4	0.94 (0.56-1.56)	3.560	0.313	15.7%
Asia	3	1.17 (0.90-1.53)	0.360	0.835	0.0%
Study design					
Population-based	2	0.92 (0.44-1.94)	2.890	0.089	65.4%
Hospital-based	4	1.16 (0.86-1.58)	1.520	0.678	0.0%
Case-control	6	1.10 (0.86-1.41)	4.750	0.447	0.0%
Cohort	1	1.06 (0.60-1.87)	-	-	-

1.10; 95%CI: 0.88-1.37; P = 0.288 for heterogeneity, I^2 = 19.2%; Table 3). The summary RR was not significant for studies that controlled for cholelithiasis[2,21,27] (summary RR = 1.28; 95%CI: 0.94-1.76; P = 0.383 for heterogeneity, I^2 = 0%; Table 3).

Alcohol consumption and risk of ECC

Six case-control studies and one prospective cohort study were identified that reported an association between alcohol consumption and the risk of ECC. The summary RR for ECC was 1.09 (95%CI: 0.87-1.37) in a random-effects model for alcoholic drinkers *vs* non-alcoholic drinkers (Figure 2B). There was no heterogeneity among studies (Q = 4.76, P = 0.575 for heterogeneity, I^2 = 0%).

Subgroup meta-analyses were conducted according to geographical region and study design. The summary RR was not significant for studies conducted outside of Asia (n = 4; summary RR = 0.94; 95%CI: 0.56-1.56; P = 0.313 for heterogeneity, I^2 = 15.7%) or in Asia (n = 3; summary RR = 1.17; 95%CI 0.90-1.53; P = 0.835 for heterogeneity, I^2 = 0%; Table 3). The summary RR was not significant for case-control studies (n = 6; summary RR = 1.10; 95%CI: 0.86-1.41; P = 0.447 for heterogeneity, I^2 = 0%) or for the cohort study (RR = 1.06; 95%CI 0.60-1.87; Table 3). The summary RRs for the population-based[19,21] (n = 2; summary RR = 0.92; 95%CI 0.44-1.94; P = 0.089 for heterogeneity, I^2 = 65.4%) and hospital-based[10,23,25,27] (n = 4; summary RR = 1.16; 95%CI: 0.86-1.58; P = 0.678 for heterogeneity, I^2 = 0%) case-control studies were not significant (Table 3).

Publication bias and sensitivity analysis

A funnel plot showed no evidence of publication bias (Figure 3). P values for Begg's adjusted rank correlation test and Egger's regression asymmetry test were 0.161 and 0.296, respectively, which indicate that publication bias probably had little effect on summary estimates.

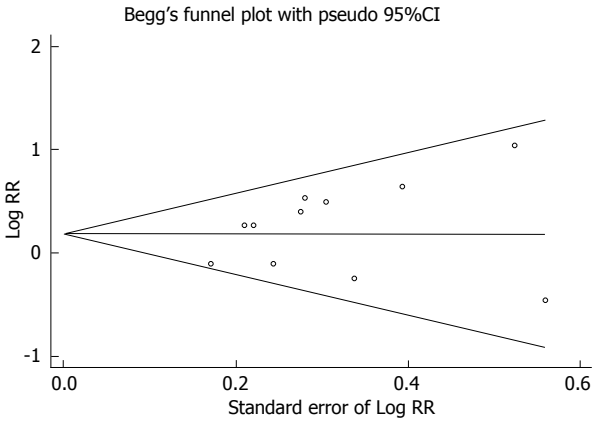

Figure 3 A Begg's funnel plot with pseudo 95% confidence limits showing the symmetrical distribution of included studies. This indicates that there was no publication bias.

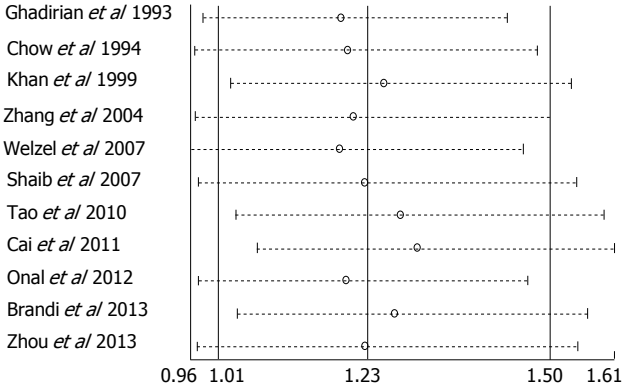

Figure 4 Influence of each individual study on the relative risks of extrahepatic cholangiocarcinoma in smokers as compared with non-smokers. Data show the RR (open circle) and 95%CI (dashed horizontal line) when the study named on the left was omitted. Random-effects estimates (exponential form) were used. RR: Relative risk.

Sensitivity analysis was performed to assess the influence of individual studies on the overall risk of ECC by excluding each individual study and recalculating the pooled RR. Similar RR and 95%CI were generated with the exclusion of each study, indicating the high degree of stability of the results (Figure 4).

DISCUSSION

In this meta-analysis, we assessed the association between smoking and the risk of ECC and between alcohol consumption and the risk of ECC. A previous meta-analysis evaluated the association between alcohol consumption and the risk of extrahepatic bile system cancer[34], and a more recent meta-analysis investigated the association between smoking and the risk of gallbladder cancer[35]. Although both of these previous studies investigated the risk of extrahepatic bile system cancer, the risk of ECC was not specified. To the best of our knowledge, this is the first study to provide comprehensive evidence of the association between smoking and alcohol consumption and the risk of ECC. In this meta-analysis we found that

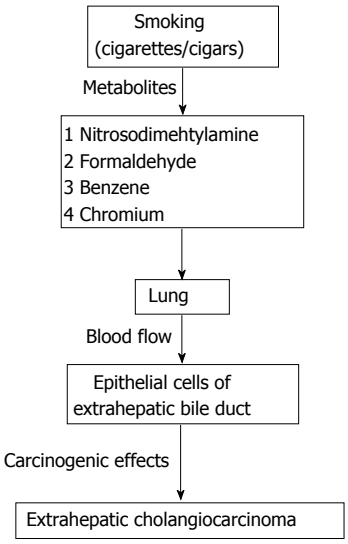

Figure 5 Proposed mechanisms by which smoking may be associated with the formation of extrahepatic cholangiocarcinoma.

smokers had a 23% increased risk of ECC as compared with non-smokers. The association between alcohol consumption and the risk of developing ECC was positive but not significant.

Although the incidence of ECC remained low among smokers and alcohol drinkers, our results carry substantial clinical and public health implications. The incidence of ECC has been on the rise worldwide in recent years, although this type of malignancy is uncommon[4]. The number of habitual smokers is rising in spite of current anti-smoking campaigns[36], and a rapid increase in the consumption of alcohol has been documented in many regions[37]. It is estimated that there are currently more than 500 million alcohol drinkers in China[16,36] and approximately 37% of Chinese adults are heavy drinkers[37].

In the subgroup analysis, we found that smoking was associated with an increased risk of ECC in non-Asian regions but not in Asia. This difference may be associated with ethnicity or with differences in the types of tobacco use between the two areas. For example, cigars contain more nicotine than regular cigarettes[38], and the pH of cigar smoke is higher than that of cigarettes, allowing more complete delivery of nicotine into the bloodstream[38,39]. Only 0.3% of the Chinese population use cigars[40], compared with 6.7% of the American population[40]. However, most of the studies included in our meta-analysis did not report the type of tobacco use, and thus we could not conduct further analysis. Although the risk of ECC was similar in smokers and non-smokers in Asia, attention should still be paid to the potential association between smoking and ECC in this population. The number of smokers in China increased from 320 to 350 million from 2005 to 2007[16,36] and 72% of Chinese citizens aged 15 years or older have been exposed to tobacco[41,42].

The potential mechanism by which smoking and alcohol consumption are associated with ECC remains unknown. Direct carcinogenic properties of smoking might be mediated by various metabolites generated in cigarettes including formaldehyde, benzene, and chromium. As early as the 1970s, it was suggested that tobacco compounds exert carcinogenic effects on the epithelial cells of the bile ducts as a result of exposure via blood flow[43] (Figure 5), and this may underlie the relation between smoking and ECC. For alcohol consumption, it is more likely that there is an indirect and bidirectional effect of carcinogenesis on the development of ECC. Moderate alcohol intake protects against gallstone formation, and gallstones are a risk factor for biliary tract cancer[17]. Metabolites of alcohol are produced in the liver and excreted into the bile duct and may interact with cholesterol metabolism. Alcohol also enhances the activation of different precarcinogenic elements[17]. Therefore, alcohol may be associated with ECC via co-effects of different mechanisms.

As with all meta-analyses of observational studies, our results have several potential limitations. First, definitions of both smoking and alcohol consumption were not consistent across the included studies. In addition, a dose-response relationship between alcohol consumption and ECC was observed in one study[10], in which the risk of ECC development was higher in heavy drinkers who consumed at least 80 g of ethanol per day. However, we could not further evaluate this dose-response relationship because of a paucity of data. The majority of studies included in this meta-analysis were case-control studies, which are more susceptible to selection and recall bias than are cohort studies. Associations between smoking or alcohol consumption and the risk of ECC in case-control studies may be confounded by changes in lifestyle after the diagnosis of ECC. In addition, 6 of the 11 case-control studies were hospital based, and these cases may not represent the general population of patients with ECC. This may have introduced selection bias into our results. Furthermore, moderate heterogeneity was observed across studies, and this may also bias the results. This heterogeneity results from diversity of the study designs, analysis of populations from different geographic locations, and the selection of participants for the different studies. These biases may distort the true associations, and data provided by this meta-analysis should thus be interpreted with caution.

Confounding effects may also have influenced the results of this meta-analysis. As noted above, moderate alcohol consumption is inversely related to gallstone disease[17,44]. When we limited the meta-analysis to studies that were adjusted for cholelithiasis, the association between smoking and ECC was no longer significant, suggesting that a confounding effect may exist. The possibility of residual confounding such as gallstone formation cannot be excluded because of a paucity of data.

Although it is possible that small studies with null results were less likely to be published than large studies with significant results, we found no evidence from funnel plot analysis and formal statistical tests for such bias.

In conclusion, the results from this meta-analysis suggest that smoking, but not alcohol consumption, is associated with a higher risk of ECC. However, the pos-

sibility that the association may be influenced by bias or confounding variables cannot be fully excluded. Further well-designed prospective studies are warranted to clarify the association between smoking and alcohol consumption and the risks of ECC.

COMMENTS

Background

Data based on epidemiological studies related to the associations between smoking and alcohol consumption and extrahepatic cholangiocarcinoma (ECC) remain conflicting. The aim of this meta-analysis was to assess the association of each risk factor with ECC.

Research frontiers

Until now, several studies have assessed the association between smoking and alcohol consumption and the risk of ECC in various regions and ethnicities; however, the results have been mixed and inconsistent. No quantitative summary of the evidence has ever been provided.

Innovations and breakthroughs

This meta-analysis identified that smoking was associated with an increased risk of ECC, especially in population-based studies and studies conducted in non-Asian regions. A positive but non-significant increased risk of ECC was observed in alcohol drinkers as compared with non-alcohol drinkers.

Applications

These results suggest that smoking is associated with an increased risk of ECC, especially in population-based studies and in studies conducted in non-Asian regions. Lifestyle changes may contribute to reducing the incidence of ECC.

Peer review

This was a well-performed meta-analysis of currently available studies on the association between smoking and alcohol consumption and ECC. The authors concluded that smoking rather than alcohol consumption may be associated with increased risk of ECC, with an emphasis on population-based studies and in non-Asian regions. This study was well designed and performed, and the results are well discussed.

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CASE REPORT

Acute cholestatic hepatitis caused by amoxicillin/clavulanate

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Abstract

Amoxicillin/clavulanate is a synthetic penicillin that is currently commonly used, especially for the treatment of respiratory and cutaneous infections. In general, it is a well-tolerated oral antibiotic. However, amoxicillin/clavulanate can cause adverse effects, mainly cutaneous, gastrointestinal, hepatic and hematologic, in some cases. Presented here is a case report of a 63-year-old male patient who developed cholestatic hepatitis after recent use of amoxicillin/clavulanate. After 6 wk of prolonged use of the drug, he began to show signs of cholestatic icterus and developed severe hyperbilirubinemia (total bilirubin > 300 mg/L). Diagnostic investigation was conducted by ultrasonography of the upper abdomen, serum tests for infection history, laboratory screening of autoimmune diseases, nuclear magnetic resonance (NMR) of the abdomen with bile duct-NMR and transcutaneous liver biopsy guided by ultrasound. The duration of disease was approximately 4 mo, with complete resolution of symptoms and laboratory changes at the end of that time period. Specific treatment was not instituted, only a combination of anti-emetic (metoclopramide) and cholestyramine for pruritus.

Key words: Hepatology; Hepatitis; Amoxicillin/Clavulanate; Drug reactions; Hyperbilirubinemia

Core tip: This report describes a case of acute cholestatic hepatitis caused by the use of amoxicillin/clavulanate. This case presented an unusual evolution, characterized by severe hyperbilirubinemia and cholestatic symptoms without the development of hepatic failure, and with total resolution requiring no specific treatment. There are few case reports in the literature that describe a similar clinical condition due to drug-induced cholestatic hepatitis.

Beraldo DO, Melo JF, Bonfim AV, Teixeira AA, Teixeira RA, Duarte AL. Acute cholestatic hepatitis caused by amoxicillin/clavulanate. *World J Gastroenterol* 2013; 19(46): 8789-8792 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i46/8789.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i46.8789

INTRODUCTION

Amoxicillin/clavulanate is a synthetic penicillin that is currently commonly used, especially for the treatment of respiratory and cutaneous infections. The addition of

clavulanate to amoxicillin provides action against bacteria that produce beta-lactamase, conferring a wide spectrum against gram-positive and -negative bacteria for the drug[1]. However, this combination considerably changes the frequency of collateral effects, as described in a study by Francesco Salvo *et al*[1] that examined the frequency of drug reactions in six Italian regions from January 1988 to June 2005. Their study showed that the percentage of gastrointestinal, hepatic and hematological reactions was significantly higher for amoxicillin/clavulanic acid (13%, 4% and 2%, respectively) than for amoxicillin (7%, 1% and 1%, respectively)[1].

With respect to hepatic side effects, cases of drug-induced hepatitis by amoxicillin/clavulanate have been reported since the 1980s, typically with a benign course. Approximately 23% of individuals on amoxicillin/clavulanate experience non-significant increases in hepatic enzymes[2]. However, a small number of severe episodes have been described, some of which are characterized by fulminant hepatitis, a disease that leads to death or the need for liver transplant[3].

Presented here is a case report of a 63-year-old male patient who developed cholestatic hepatitis after use of amoxicillin/clavulanate.

CASE REPORT

The male, a 63-year-old patient was admitted to the Hospital Renascentista on September 1, 2012, with a history of jaundice, choluria, fecal acholia, generalized pruritus, malaise, hyporexia and sporadic nausea without associated vomiting for five days.

The patient had hypertension for 10 years, dyslipidemia for 3 years, a recent diagnosis of altered fasting blood sugar, oligosymptomatic benign prostatic hyperplasia for 3 years and was overweight. He took 50/12.5 mg of atenolol/chlorthalidone once a day. He indicated that it had been approximately 45 d since he had used a topical corticoid for 15 d for acute otitis, and denied using any other medications. He had no history of trauma or surgery, and no epidemiological history of note. He was a non-smoker and drank alcohol on the weekend (three cans of beer on Saturdays and Sundays), but did not drink in the periods preceding the beginning of the symptoms.

Upon physical examination at admission, the patient was jaundiced (2+/4) with small traumatic lesions on the skin, was afebrile, normotensive (AP: 130/80 mmHg) with a heart rate of 80 beats/min, eupneic without changes in pulmonary auscultation and showed normal findings on abdominal examination with no visceromegaly.

The admission tests indicated Hb: 130.6 g/L, leukocytes: 7200 cells/mm3 (normal differential), platelets: 248000 cells/mm^3, fasting glycemia: 1100 mg/L, creatinine: 9 mg/L, urea: 290 mg/L, Na: 137 mEq/L, K: 3.8 mEq/L, Mg: 18 mg/L, Ca: 42 mg/L, blood gases: normal, CRP: 66 mg/L, amylase: 40 IU/L, lipase: 35I U/L, AST: 78 IU/L, ALT: 200 IU/L, alkaline phosphatase: 60 IU/L, GGT: 33 IU/L, albumin: 33 g/L, complete coagulation profile: normal, LDH: 2 94I U/L, total bilirubin: 83 mg/L, direct bilirubin: 50.1 mg/L, reticulocytes: 0.9%, haptoglobin: 1440 mg/L (160-2200 mg/L).

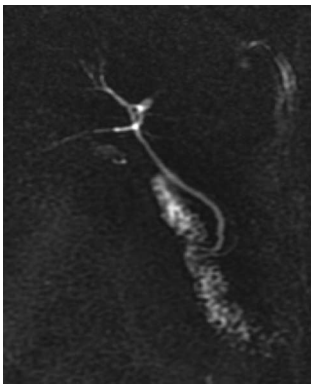

Figure 1 Magnetic resonance of abdomen and bile duct-nuclear magnetic resonance. Images of bile duct-nuclear magnetic resonance demonstrating the biliary tree without evidence of obstructive processes.

Diagnostic investigation began with ultrasonography of the upper abdomen, which demonstrated only cholesterolosis of the biliary vesicles. Serological tests for hepatitis A, hepatitis B, hepatitis C, hepatitis E, cytomegalovirus, Epstein-Barr, dengue, leptospirosis and HIV were all negative. Auto-immune analysis demonstrated negative anti-nuclear factor, anti-smooth muscle and anti-mitochondria, and normal serum IgG and serum IgM. Nuclear magnetic resonance (NMR) of the abdomen with bile duct-NMR was subsequently requested, which demonstrated constricted biliary vesicles (Figure 1).

The Council for International Organizations of Medical Sciences (CIOMS) score was +9 points and the Clinical Diagnostic Scale (CDS) score was +11 points.

During hospitalization, the patient developed a progressive worsening of hyperbilirubinemia (Table 1), pruritus, malaise and nausea, where he received symptomatic treatment with cholestyramine 4 g four times per day and metoclopramide when necessary.

Because there was no clinical improvement on day 30, the patient was more extensively questioned. Additionally, the accompanying family members were asked to bring all recent medical documents. A prescription was found for amoxicillin-clavulanate 500 mg 3 times a day for twenty-one days, which had been initially used forty-five days ago, together with a topical corticoid to treat acute otitis. The presence of these drugs led to a suspected diagnosis of drug-induced cholestatic hepatitis, which was confirmed by transcutaneous liver biopsy (Figure 2).

The patient began to show improvement, both clinically and based on laboratory results, after thirty days of hospitalization and was discharged with the use of cholestyramine 4 g four times per day, and for recommendation of outpatient follow-up.

Four months after the onset of symptoms, he became asymptomatic with jaundice and other previous changes resolved, normal routine liver tests, and he began receiving only previous chronic anti-hypertensive therapy.

Table 1 Results of routine hepatic tests during hospitalization

	9/1	9/3	9/6	9/9	9/10	9/11	9/18	9/22	9/26
AST (IU/L)	78	89	80	75	51	50	63	77	70
ALT (IU/L)	200	245	160	130	69	59	65	89	82
BT/BD (mg/L)	80.3/50.1	100.9/70.0	110.3/70.9	150.6/90.0	180.3/110.0	210.9/140.0	250.9/160.0	280.9/180.0	310.2/200.0
AP (IU/L)	60	80	110	140	193	188	239	300	311
GGT (IU/L)	33	65	90	123	150	134	175	223	226
INR	1.01	1.00	1.12	1.1	1.0	1.2	1.0	1.0	1.0

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; BT/BD: Total bilirubin/Direct bilirubin; AP: Alkaline phosphatase; GGT: Gamma-glutamyl transpeptidase.

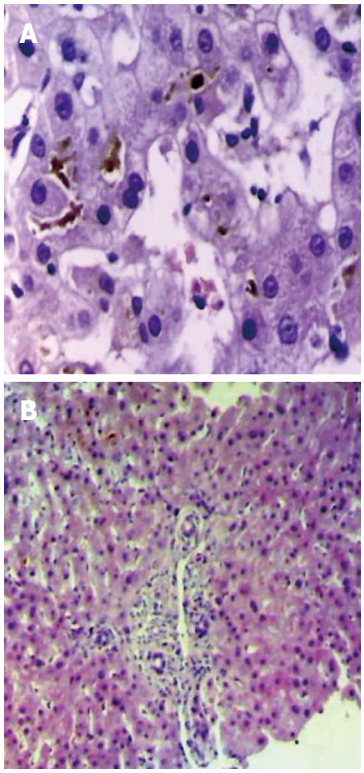

Figure 2 Transcutaneous liver biopsy guided by ultrasound. A: Liver biopsy at x 100 magnification - moderate cholestasis is demonstrated associated with discrete parenchymal activity with slight tumefaction of hepatocytes; B: Liver biopsy at × 40 magnification - portal space is shown with discrete increase in periportal lymphocytes, extravasation of lymphocytes towards the interface (spillover), absence of piecemeal necrosis and cholestasis.

DISCUSSION

Amoxicillin/clavulanate is a widely used antibiotic that is associated with adverse effects, especially of the cutaneous, gastrointestinal, hepatic and hematologic types[1]. The incidence of hepatic damage by amoxicillin/clavulanate is greater than that associated with amoxicillin administration alone (1.7 *vs* 0.3 for every 10000 prescriptions)[1-4]; predominantly cholestatic lesions, although isolated mixed and hepatocellular lesions also occur[2,5-7]. There are also reports in the literature of patterns of granulomatous lesion secondary to the use of the medication in question[8].

Histopathological examination usually reveals centrilobular or panlobular cholestasis and inflammation, predominantly lymphocytic, portal and periportal, with neutrophils and eosinophils frequently present[2,3,6]. Other biopsy findings include degeneration and necrosis of ductal epithelial cells, ductopenia[4] and vacuolization and necrosis of hepatocytes[5,6], all in addition to granulomatous inflammatory process[8].

The pathogenic events that cause lesions due to the use amoxicillin/clavulanate require further study[3], but it is believed that idiosyncratic immunoallergic mechanisms are the underlying causes[3,6,7,9-11]. The common presence of eosinophils in the inflammatory infiltrate[3], the co-existence of manifestations of hypersensitivity, such as skin rash and hypereosinophilia[3], the documentation of the involvement of specific autoantibodies (anti-mitochondrial type 6, anti-LKM2 and anti-LM antibodies)[3] and class II HLA antigens (DRB1*1501-DRB5*0101-DQB1*0602)[11] reinforce the hypothesis that immune aggression is involved in the lesions formed due to amoxicillin-clavulanate use.

The risk factors for hepatotoxicity caused by amoxicillin/clavulanate include male sex, associated alcohol consumption, repeated courses of the drug, concomitant consumption of other hepatotoxic substances[2] and age over 55 years[7]. Treatment duration has been included as a predisposing factor in some reviews[3].

The clinical characteristics are predominantly cholestatic signs and symptoms, which include malaise, hyporexia, nausea, vomiting, jaundice, choluria, fecal acholia, cutaneous pruritus and, less commonly, painful hepatomegaly. Manifestations associated with hypersensitivity can occur, such as skin rash and fever, with an incidence as high as 50%[12,13]. The symptoms can begin in any period after the end of treatment, but typically appear between 4 and 10 wk and are self-healing, as they are resolved in 4-16 wk. Reports of chronification, as described by Ryley *et al*[14], are extremely rare.

Severe hyperbilirubinemia, changes in laboratory liver function blood tests and neurological alterations constitute the criteria for a poor prognosis, with the possibility of the development of fulminant hepatitis[3,6,9].

Treatment consists mainly of support and should attend to various aspects of hepatic lesions. It is common for patients to become dehydrated due to decreased fluid intake and vomiting. Therefore, the evaluation of the volemic status is essential and should be corrected rapidly if necessary. Additionally, the cholestatic symp-

toms can become limiting and require prescriptions for symptomatic patients, such as anti-emetics and analgesics, in addition to medications to control pruritus. Generally, cholestyramine, anti-histamines, ursodeoxycholic acid and sertraline are used dependent on the intensity of symptoms and the experience of the service with the use of the drugs.

Due to the likely immunological mechanism of hepatic lesions, including hypersensitivity reactions mediated by eosinophils, some authors advocate the use of a systemic corticoid in the treatment of severe cases and in those with potential severity, such as hyperbilirubinemic individuals[6]. However, there is no evidence of reduced morbidity.

This study reported the case of a 63-year-old patient who began to show signs of cholestatic icterus after 6 wk of prolonged use of amoxicillin/clavulanate. The patient developed severe hyperbilirubinemia, but did not meet other criteria of severity. The time of disease was approximately four months, with complete resolution of symptoms and laboratory changes. The patient did not receive any specific treatment, only a combination of anti-emetics (metoclopramide) and cholestyramine for pruritus.

Of the risk factors for hepatotoxicity due to the drugs, advanced age, male sex, alcohol drinking and prolonged antibiotic therapy were all present in this case. The period of the onset of symptoms, the clinical characteristics and the time for complete recovery were in accordance with other reported cases. The degree of hyperbilirubinemia is important in the laboratory profile, with few described cases of total bilirubin reaching values higher than 300 mg/L[6]. Although there is a possible relation with severity, our patient did not show any signs of hepatic dysfunction. A complete history was taken, as described, to rule out other causes of hepatotoxicity. Based the Council for International Organizations of Medical Sciences score (CIOMS) (+9 points - very likely association)[15], the Clinical Diagnostic Scale (CDS) score (+11 points - possible association)[16] and information from liver biopsy, this case had a high probability of hepatotoxicity due to drug use.

The biopsy findings, besides the cholestasis, were typical of hepatitis due to amoxicillin-clavulanate, periportal lymphocytic inflammation with damage to hepatocytes, alterations found in the pathology[2-6].

A relevant factor in the case, which made the diagnosis difficult, was the denial of the patient to taking the medication. Thus, this report demonstrates the importance of thorough anamnesis and careful verification for antibiotics and/or other drugs use.

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CASE REPORT

Pancreatic solid cystic desmoid tumor: Case report and literature review

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Author contributions: Zhu LH and Xu B performed the operation; Wu JG and Ni JJ collected data; Wang XF supervised the surgery; Zhu LH, Xu B and Matro E wrote the paper.
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Abstract

Desmoid tumors (DTs) are nonmetastatic, locally aggressive neoplasms with a high rate of postoperative recurrence. Pancreatic DTs are especially rare; only a few cases have been reported to date. This paper describes a case of a sporadic cystic DT of the pancreas managed successfully with central pancreatectomy, with no signs of recurrence 40 mo after surgery. According to the literature, this is the first reported case in China of a pancreatic DT presenting as a solid cystic lesion, as well as the first pancreatic DT managed with central pancreatectomy and pancreaticogastrostomy. We report the case for its rarity and emphasize disease management by concerted application of clinical, pathological, radiological and immunohistochemical analyses.

Key words: Pancreatic tumor; Desmoid tumor; Cystic tumor; Central pancreatectomy; Pancreaticogastrostomy

Core tip: Desmoid tumors (DTs) are rare, representing approximately 0.03% of all tumors and 3% of soft tissues tumors. They are nonmetastatic and locally aggressive, with a high local recurrence rate. The pancreas is an extremely rare location for DTs. Moreover, pancreatic desmoids resembling solid cystic tumors are the rarest form of DTs. We report a 17-year-old patient presenting with a sporadic cystic DT of the pancreas, and subsequent disease management with central pancreatectomy. We report the case for its rarity and emphasize disease management by concerted application of clinical, pathological, radiological and immunohistochemical analyses. Associated English-language literature is also reviewed and summarized.

Xu B, Zhu LH, Wu JG, Wang XF, Matro E, Ni JJ. Pancreatic solid cystic desmoid tumor: Case report and literature review. *World J Gastroenterol* 2013; 19(46): 8793-8798 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i46/8793.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i46.8793

INTRODUCTION

Desmoid tumors (DTs), also known as musculo-aponeurotic fibromatoses, are locally aggressive soft tissue neoplasms histologically characterized by fibroblastic proliferation within a collagen matrix[1]. Intra-abdominal DTs are associated with familial adenomatous polyposis (FAP) and Gardner syndrome, and they may also occur sporadically or subsequent to localized trauma (surgical or non-surgical). DTs possess negligible metastatic potential and frequently remain asymptomatic for extended periods of time before a diagnosis can be made on the basis of either vague, chronic symptoms or obvious mechanical complications. Aggressive expansion into adjacent tissues

results in significant morbidity due to nerve and organ damage, and the mortality rate due to DTs approaches 10%. DTs also present a considerable dilemma for clinicians because of their high rates of recurrence after surgery, especially following conservative resection. The pancreas is a rather rare location for DTs, even though sporadic DTs are found within the pancreas more frequently than sporadic intra-abdominal DTs[2]. Since 1956 when Wilson reported the first case of a pancreatic DT resembling a pancreatic pseudocyst, only a few similar cases have been reported[1,3].

Here, we report a 17-year-old patient presenting with a sporadic cystic DT of the pancreas, and subsequent disease management with central pancreatectomy. Pertinent English-language literature is also reviewed and summarized.

CASE REPORT

In June 2009, a 17-year-old boy presented to the gastroenterology department in our hospital with complaints of upper abdominal pain, nausea and vomiting for 5 d. He had not had passage of stools for 2 d. He denied hematemesis, melena, diarrhea, weight loss or fever. Past medical and surgical history was unremarkable and he denied any abdominal trauma. Abdominal auscultation revealed no abnormalities and percussion elicited splashing sounds in the upper abdominal region. A mass of 6 cm × 5 cm was palpated in the left upper quadrant without tenderness or rebound tenderness. Neither abdominal muscle guarding nor enlarged superficial lymph nodes were noted. Admission laboratory data were within normal limits, and serum tumor marker levels [carcinoembryonic antigen (CEA) and cancer antigen (CA) 19-9] were not elevated. Abdominal ultrasonography demonstrated a solid cystic mass within the body of the pancreas (Figure 1). Abdominal plain X-rays revealed no apparent signs of bowel obstruction, while gastroscopy demonstrated intragastric fluid retention with luminal compression from a mass within the outer gastric wall (Figure 2). Abdominal computed tomography (CT) with intravenous contrast delineated a cystic mass within the central pancreas, invading the horizontal part of the underlying duodenum (Figure 3). Additionally, endoluminal ultrasonography (EUS) demonstrated a cystic hypoechoic tumor posterior to the stomach of 6.7 cm × 5.5 cm and containing four cystic cavities (Figure 4). Examination of EUS-guided fine needle aspirate only demonstrated inflammatory tissue; however, cystic fluid examination revealed elevated levels of both CEA and CA19-9, at 1378 and 425.4 ng/mL, respectively. In light of the aforementioned pancreatic mass and resultant duodenal obstruction, the patient was transferred to the Department of General Surgery for emergency exploratory laparotomy.

During the surgery, a solid cystic mass of 8.6 cm × 6.0 cm within the pancreatic neck and body invading the adjacent horizontal portion of the duodenum was noted (Figure 5). The mass was adjacent to the posterior wall of the stomach. No regional lymphadenopathy was noted. The tumor was primarily located within the central pancreas, therefore, central pancreatectomy was deemed in order, accompanied by partial distal stomach and horizontal duodenal *en bloc* resection (Figure 6). Digestive tract reconstruction was performed *via* pancreaticogastrostomy, duodenojejunostomy (side to side) and gastrojejunostomy (Billroth Ⅱ, Figure 7). The postoperative course was uneventful; total parenteral nutrition was provided for 7 d after surgery and the patient was discharged on postoperative day 12. Follow-up examinations with CT, gastroscopy, gastrointestinal imaging and colonoscopy were performed once every 6 mo. The patient recovered well with no complaints of discomfort, malnutrition, or bowel movement abnormalities and continued his study in school as previously. CT showed no recurrence 20 mo postoperatively (Figure 8).

Pathological examination identified a pancreatic DT with several cystic cavities and adjacent duodenal invasion. Histological sections of the solid tumor showed proliferation of spindle-shaped or stellate cells, growing in fasciculate and storiform patterns within a myxoid intercellular matrix. The cystic lesion was lined predominantly by chronically inflamed fibrous tissue and small areas of benign columnar epithelium. Specimen surfaces from the stomach and duodenum contained a mesenteric plaque composed of well-defined sheets of densely collagenized fibrous tissue, which entrapped fat, blood vessels, nerve fibers, and smooth muscle. In the areas between the solid tumor and pancreatic parenchyma, the mesenteric plaque merged with the desmoid masses, entrapping pancreatic acini and resulting in irregularly dilated ducts. The cystic area was the result of dilatation of entrapped excretory pancreatic ducts (Figure 9). Subsequent immunohistochemical analysis found smooth muscle actin and cytokeratin to be positive, while desmin, CD117 (c-kit), CD34, S-100, calretinin and estrogen receptor (ER) were negative.

DISCUSSION

DTs are rare and first appeared in the medical literature in the early 19th century. The term is derived from the Greek "desmos", describing a band- or tendon-like attribute. DTs represent approximately 0.03% of all tumors and 3% of soft tissues tumors[4]. They are nonmetastatic and locally aggressive, with a high local recurrence rate, and may arise virtually anywhere within the body. The tumors are benign and are characterized by the absence of pleomorphism, atypia, or hyperchromatic nuclei[5]. Desmoids are particularly predisposed to muscular fascia, but may occur at any fascia. The most frequent sites for these tumors are the torso and extremities. Recent clinical studies have demonstrated that 37%-50% of DTs arise in the abdominal region[4,6,7].

DTs have recently been classified depending on their point of origin as extra-abdominal, abdominal and intra-abdominal; the latter type further subclassified into mes-

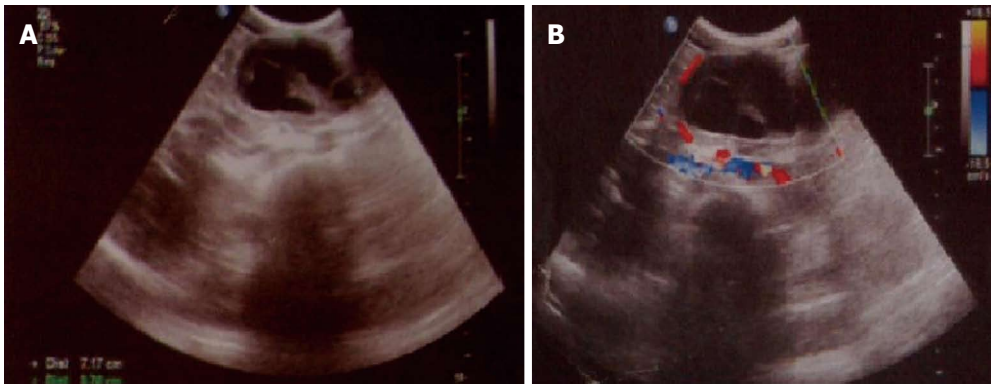

Figure 1 Abdominal ultrasonography demonstrated a solid cystic mass within the body of the pancreas. A: Ultrasonography showed a solid cystic mass within the body of the pancreas; B: Ultrasonography showed that the mass was surrounded by blood vessels.

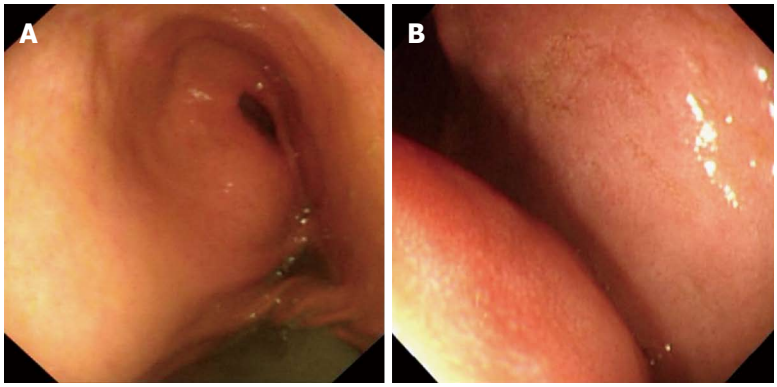

Figure 2 Gastroscopic imaging demonstrating fluid within the stomach and wall compression caused by an external mass. A: Gastroscopy demonstrated intragastric fluid retention; B: Stomach wall compression by an external mass.

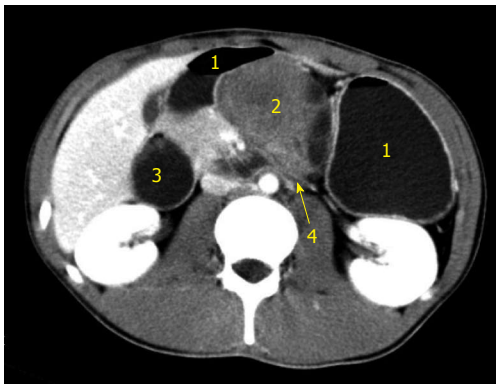

Figure 3 Abdominal computed tomography with contrast (venous phase) demonstrating a solid cystic mass invading the horizontal portion of the duodenum. 1: Stomach; 2: Pancreatic cystic mass; 3: Enlarged duodenum; 4: Horizontal duodenal portion invaded by the tumor.

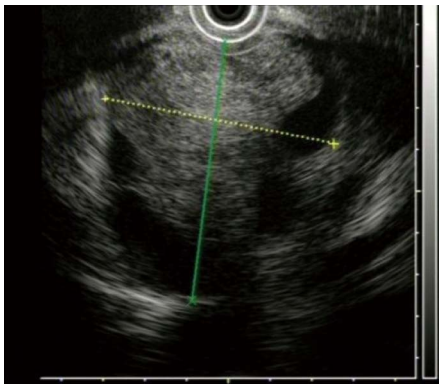

Figure 4 Endoscopic ultrasonography revealing a pancreatic cystic hypoechoic tumor located posterior to the stomach, with four cystic cavities, about 6.7 cm × 5.5 cm in dimension.

enteric and pelvic fibromatoses[8]. Associations between abdominal desmoids with FAP and Gardner syndrome have been well documented, suggesting a genetic predisposition to such lesions. Abdominal desmoids occur more frequently in FAP patients, with an incidence of 3.5%-32%, while in the original Gardner syndrome, the incidence was 29%[4,9]. In both FAP and familial non-FAP tumors, mutation of the adenomatous polyposis coli (APC) gene on the long arm of chromosome 5 has been implicated as the primary causative mechanism. As a result of APC mutation, β-catenin degradation markedly decreases, promoting fibroblastic proliferation *via* nuclear signaling pathways[10].

DT etiology has not been well defined. A history of trauma to the site of the tumor, often surgical in nature, may be elicited in approximately 25% of cases[11,12]. Anecdotal evidence of tumor regression during menopause[13],

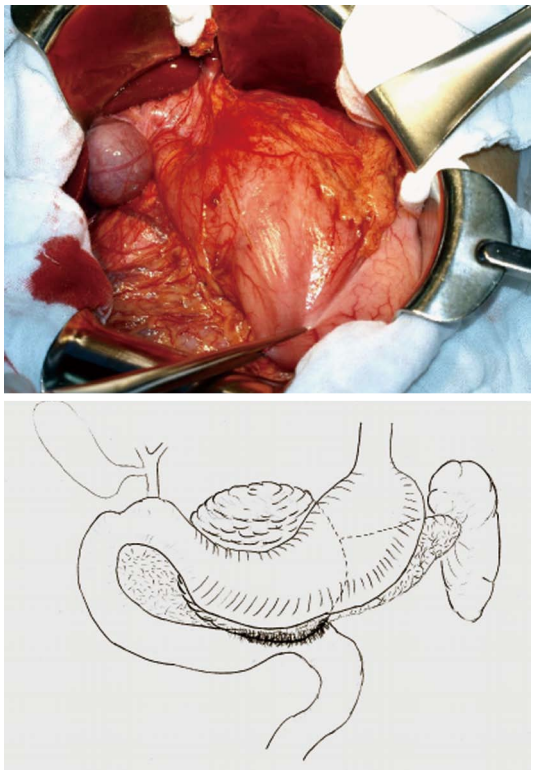

Figure 5 Exploratory laparotomy revealed a cystic mass of size 8.6 cm × 6.0 cm within the pancreatic neck and body invading the horizontal duodenum.

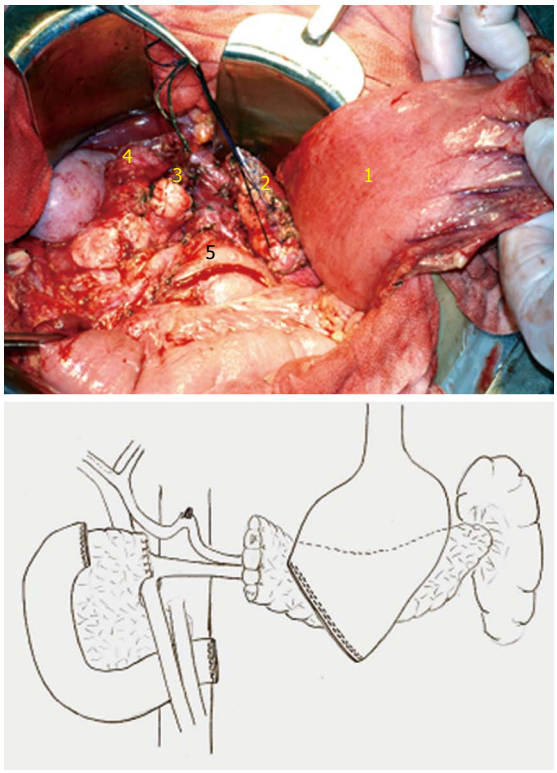

Figure 6 The central pancreas (containing the tumor), distal stomach and duodenal portion were all resected. 1: Stomach; 2: Left resected end of pancreas; 3: Right resected end of pancreas; 4: Upper resected end of duodenum; 5: Lower resected end of duodenum.

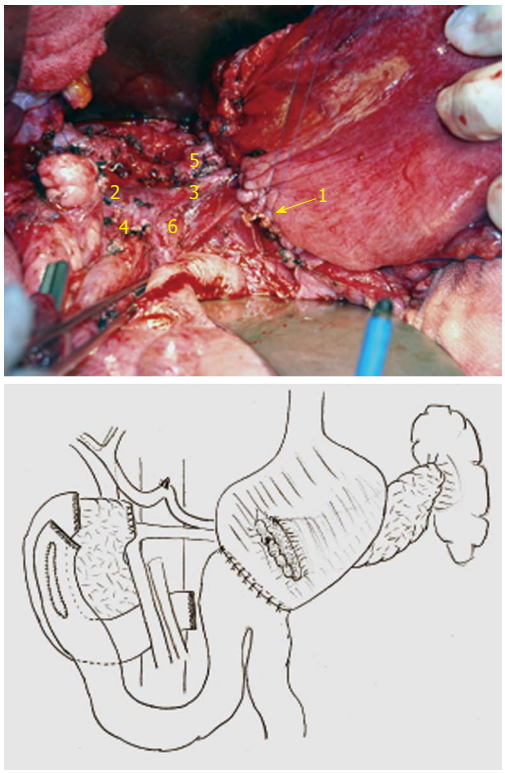

Figure 7 Reconstruction was performed *via* pancreaticogastrostomy, duodenojejunostomy (side to side) and gastrojejunostomy (Billroth Ⅱ). 1: Pancreaticogastrostomy; 2: Portal vein; 3 Splenic vein; 4: Superior mesenteric vein; 5: Splenic artery; 6: Superior mesenteric artery.

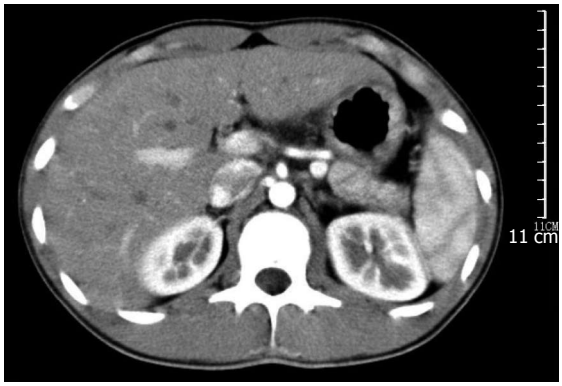

Figure 8 Contrast computed tomography examination (arterial phase) demonstrated no recurrence 18 mo after operation.

the development of desmoids in patients taking oral contraceptives[14], and reports of tumor regression with tamoxifen treatment[15] underline the apparent role of estrogen in the multifactorial pathogenesis of the neoplasm.

The pancreas is an extremely rare location for DT manifestation. Moreover, pancreatic desmoids resembling solid cystic tumors are the rarest form of DTs. Most relevant literature concerns FAP-associated desmoids; only nine cases of pancreatic DTs have been described in the English literature to date[2,16-22]. Although intra-abdominal DTs are widely considered to be associated with FAP,

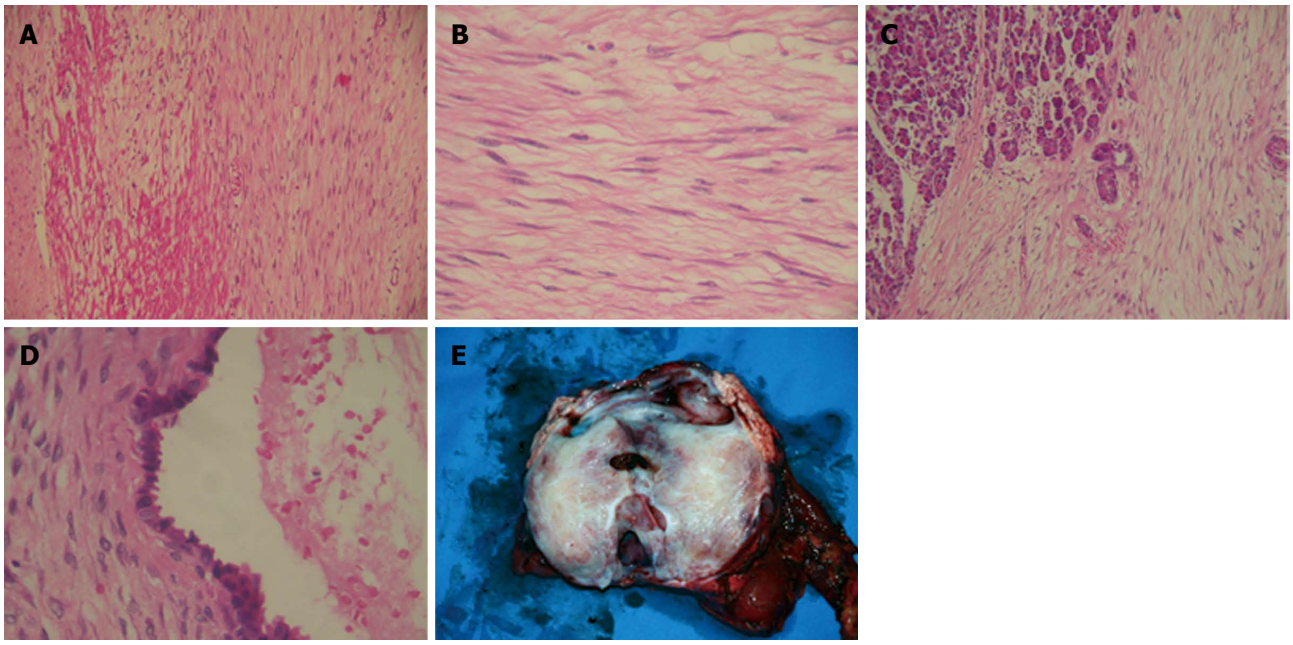

Figure 9 Pathological examination. A: Pancreatic desmoid tumor with invasion to the duodenal wall; B: Proliferation of spindle-shaped stellate cells in fasciculate and storiform growth patterns within a myxoid intercellular matrix; C: Pancreatic infiltration by the tumor; D: The cystic area resulted from dilatation of entrapped excretory pancreatic ducts; E: Gross view of resected tumor and pancreas.

such association was noted in only one of the 10 reported cases (including our present case) of pancreatic DTs. Pancreatic DTs occurring after pancreatic surgery or biopsy were noted in three of the 10 reported cases[18,19]. Presenting symptoms consisted mainly of epigastric pain (6/10) and weight loss (4/10). The pancreatic DTs were mostly localized to the tail (6/10) and measured 15-85 mm in length.

In the present case, the patient was initially diagnosed with a mucinous cystic or solid pseudopapillary neoplasm. These diagnoses were suggested due to the cystic characteristics of the tumor, and pathological examination revealed the cystic components bearing a resemblance to retention cysts. Only two previously published cases of pancreatic DTs presented as cystic tumors[21]. In the first case, the cystic component corresponded to a benign retention cyst. The other case corresponded to an intraductal papillary mucinous neoplasm situated adjacent to a DT, but lacking true intralesional cystic components[19].

Differentiating DTs from other types of soft tissue neoplasms may be difficult based on histological analyses alone. Expression was negative for CD34, CD117, S-100 and desmin, which excluded a gastrointestinal stromal tumor, solitary fibrous tumor, schwannoma, leiomyoma and leiomyosarcoma, respectively[23,24]. DTs are theoretically associated with clonal myofibroblastic proliferation and somatic mutation of the Wnt/β-catenin gene, leading to the intranuclear accumulation of β-catenin. Recent efforts in immunostaining of intranuclear β-catenin and the gene responsible for its mutation proved efficient in discriminating DTs from other benign and malignant fibroblastic and myofibroblastic lesions[25].

In the case of intra-abdominal DTs, surgical resection is generally performed in the event of extensive tumor invasion and potentially life-threatening complications. However, resection is usually a difficult procedure because of considerable vascular involvement among the mesenteric and retroperitoneal areas. In this case, the tumor was located in the pancreatic neck and body, invading the horizontal portion of the duodenum and the posterior stomach wall. In order to perform resection with a tumor-free margin, central pancreatectomy with accompanying *en bloc* resection of the distal stomach and horizontal duodenal portion was deemed appropriate (Figure 6). Digestive tract reconstruction mandated three anatomoses for continuity maintenance (Figure 7).

Intra-abdominal DTs possess high rates of local recurrence after surgical resection, particularly in patients with FAP or Gardner syndrome. Intriguingly, frequent recurrences appear to be absent in cases of sporadic pancreatic DTs, according to the limited number of follow-up reports available[2,16-22]. A single reported case of recurrence was noted in a patient with FAP by Pho *et al*[21]. The present case remains disease free 40 mo after surgery, consistent with previous reports.

In summary, the pancreas remains a rare location for DTs. Pancreatic DTs presenting as solid cystic tumors are the rarest form of desmoids. There are no notable clinical symptoms, tumor markers or imaging features to aid in diagnosis. Nuclear immunostaining of the β-catenin protein and its corresponding coding gene are efficient in distinguishing DTs from other lesions. Cytotoxic treatments may be utilized for either unresectable neoplasms or those unresponsive to more benign treatment, as radiotherapy may be limited in use due to extensive bowel

geography. Radical surgical resection with tumor-free margins appears to produce an excellent prognosis when applicable.

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In press

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.00000 35706.28494.09]

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]

No author given

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]

Issue with no volume

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Personal author(s)

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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13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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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

Electronic journal (list all authors)

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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