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EDITORIAL

Estrogen, estrogen receptors, and hepatocellular carcinoma: Are we there yet?

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Abstract

A protective role of the sex steroid hormone estrogen in hepatocellular carcinoma (HCC) was suggested a few decades ago according to clinical data showing higher HCC morbidity and mortality among males. Several recent studies further confirmed the anti-cancer effects of estrogen in the liver. However, it remains to be identified how to exploit estrogen signalling within clinical settings for HCC treatment. There are several unresolved issues related to the estrogen pathway in liver cells. The main problems include the absence of a clear understanding of which estrogen receptor (ER) isoform is predominantly expressed in normal and malignant liver cells, the ER isoform expression difference between males and females, and which ER isoform should be targeted when designing HCC therapy. Some of those questions were recently addressed by Iyer and co-authors. The current editorial review critically analyses the study by Iyer *et al* (*WJG*, 2017) that investigated the expression of ER subtypes in liver samples collected from patients with a healthy liver, hepatitis C virus cirrhosis, and HCC. ER presence was evaluated in association with gender, intracellular localization, inflammation marker NF-κB, and proliferation-related effector cyclin D1. The study limitations and advantages are discussed in light of recent advances in the HCC and estrogen signalling areas.

Key words: Hepatocellular carcinoma; Hepatitis C virus; Hepatitis; Estrogen receptors; Cirrhosis

Core tip: Recent discoveries confirmed that the female sex hormone estrogen protects against the development and progression of hepatocellular carcinoma (HCC). However, the mechanism of estrogen's anti-oncogenic effects and the specific impact of estrogen receptor (ER) signalling in HCC are unclear and controversial. It is essential to determine how to exploit the estrogen signalling pathway within a clinical setting for HCC

treatment. The current editorial review critically analyses the Iyer *et al* (*WJG*, 2017) study that investigated the expression of ER subtypes in liver samples collected from patients with a healthy liver, hepatitis C virus cirrhosis, and HCC.

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INTRODUCTION

Despite considerable advances in the treatment of various malignancies, there are still limited cure options for hepatocellular carcinomas (HCC), a high-lethality malignancy. Determined as a possible outcome of the chronic liver diseases with cirrhosis, HCC was strongly linked to hepatitis B and C, alcoholic liver disease, and non-alcoholic steatohepatitis[1]. As a preventive factor, the role of steroid hormones in the regulation of hepatic malignant transformation was suggested after a consistent gender disproportion was observed in the incidence of HCC worldwide. Females, at the premenopausal age when circulating estrogen is high, are protected from HCC and get better recovery after HCC treatment[2,3]. However, the mechanism of the hormone anti-oncogenic signaling and the specific impact of estrogen receptor (ER) isoforms in HCC development and progression are unclear and controversial.

The precedent is based on the high variability of the tested samples and low significance of the established association between ER levels and disease-specific outcome shown in several studies[4,5]. Consequently, clinicians are in understandable disagreement about the potential benefits of hormonal therapy in HCC patients. Nevertheless, the hypothesis about the protective impact of estrogen signaling pathway against HCC was confirmed by several groups[4,5]. The worldwide epidemiological data demonstrate the strong and consistent prominence of HCC among men, indicating estrogen-dependent protection against liver cancer that deserves serious consideration. Indeed, there is an urgent need for a detailed investigation of ER expression and signaling in normal liver and HCC, accenting the fact that HCC is a leading cause of cancer-related death with growing incidence worldwide. It is pleasing to see that Iyer *et al*[6] addressed the problem using a few novel approaches.

STUDY ANALYSIS

HCC is a disease with multifactorial causes and genetic variability. Although the liver is mostly considered an accessory organ of the digestive system, it is also a hormone-sensitive organ and therefore is influenced by gonadal hormones, such as estrogen. The hypothesis about the regulatory role of estrogen and ER in the development and progression of HCC inspired Iyer *et al*[6] to test the level of ER expression in liver tissues from normal subjects and patients with chronic hepatitis C virus (HCV)-related cirrhosis and HCC. The authors analysed the respective association of ER isoforms with inflammatory and oncogenic markers of HCC pathogenesis. The expression of ER subtypes, ERα and ERβ, was thoroughly evaluated at the mRNA and protein levels in relation to gender and type of disease[6]. The history of detection of ER variants expression requires special explanation. For several decades due to an absence of variant-specific antibodies and a lack of knowledge about the role of these variants in carcinogenesis, the level of ER expression was evaluated using non-specific antibodies that recognized either both ERα and ERβ variants unseparated, or identified only ERα, thus neglecting ERβ. This is not surprising, as the ERβ isoform was only identified in 1996.

Obviously, the data received with the use of non-specific antibodies should be considered carefully and should not be used for generalization. Furthermore, the data received with the use of only one type of ERα-specific antibodies that allows detection of one known ERα subtype should be considered partial, as there are several variants of ERα and ERβ currently known with quite different tissue-specific expression patterns and functioning[7,8]. The wisely-designed and isoform-specific investigation of ER variant expression in liver samples becomes more complicated, time-consuming, and expensive, thus it is only affordable for large, well-financed, clinical laboratories. Unfortunately, this kind of comprehensive variant-specific analysis has not yet been conducted, while most of ER-HCC studies remain inconclusive, as they are based on a small sample size with the use of at most two types of ER isoform-specific antibodies.

Small sample size is one of the major problems of nearly all investigations aimed to determine the level of ERs in gender-specific settings for diseases of gastro-intestinal and accessory organs, including the liver and pancreas. As stated above, females are less susceptible to HCC and, as a result, women's liver samples are often under-represented in small studies. Similarly, Iyer *et al*[6] could not perform statistically relevant gender analyses due to the limited size of the patient cohort and the scarcity of female samples in the diseased groups. This problem could be overcome in a study conducted by two or more collaborating clinical laboratories that register a sufficient number of female patients with HCC.

The study determined significantly higher expression of ERα and ERα:ERβ expression ratio in normal (healthy) males as compared to females. The findings demonstrated by Iyer *et al*[6] suggest some potential

predisposition of males to develop liver cancer as increased *ER* gene expression was previously shown in liver tumours from HCC patients[9], and was linked to higher proliferation rate in other cancers. Iyer *et al*[6] detected an increase in the liver mRNA expression of ERα (ESR1) and ERβ (ESR2) subtypes in chronic HCV and HCV-related HCC as compared to normal. However, in contrast to healthy liver, ERα (ESR1) mRNA transcriptional levels were decreased in the male liver with chronic HCV and HCV-related HCC as compared to normal liver samples. The study included premenopausal female subjects (female controls age range 42-67) indicating that at least some of them had a reasonably high level of circulating estrogen. However, the HCV and HCC groups included only menopausal and post-menopausal females suggesting that the level of circulating estrogen in those females is comparable to men. Unfortunately, the real level of circulating estrogen was not measured in any of those groups.

Considering the applied methods, the authors (Iyer *et al*[6]) evaluated the amount of ERα and ERβ proteins using the western blotting technique and immunohistochemistry (IHC) on paraffin-embedded tissue samples. The latter approach allows for the observation of intracellular localization of the receptor variants. Many studies addressed the mechanisms of the ER signaling pathways in cytoplasm and nuclear compartments, but those studies were mostly assessing breast carcinomas. The findings related to ER intracellular localization in breast tissues might be irrelevant to liver samples, although indicate the necessity of further investigations. To confirm the IHC data, ER isoform localization was assessed using subcellular fractionation. The analysis indicated some favorable tendency towards nuclear translocation of ERα and ERβ proteins in HCV and HCC samples. The increased nuclear-to-cytoplasmic ratio and dominant presence of both of ER variants in the nuclear space of cells from the diseased groups suggest ER-related gene activation.

Iyer *et al*[6] assessed activation of an oncogenic marker cyclin D1 and found the increased expression of the protein in the nuclear and cytoplasmic fractions of diseased livers. Increased cyclin D1 level is supposed to stimulate hepatocyte proliferation during chronic HCV-infection. The expression of nuclear ERα and ERβ positively correlated with nuclear cyclin D1 in both HCV related cirrhosis and HCV-related HCC. The data suggests that ER-subtypes stimulate the activation of transcriptional activity during the progression of the disease towards malignancy. However, this effect and related ER gene activation require further confirmation on the level of established ER target genes including progesterone receptor and cathepsin D; although activation of ER target genes in the liver has not been well-studied.

Besides proliferation-related effector, the authors analysed activation of the inflammation-related signalling pathway of NF-κB. Although NF-κB activation was associated with ER signalling, the link is very controversial and NF-κB also mediated anti-inflammatory effects of ER[10]. Considering evidence confirming the inflammation-related role of NF-kB in the liver[11], it is not surprising that Iyer *et al*[6] detected a significantly higher expression of phosphorylated NF-κB in chronically infected livers with HCV and in HCC tissue samples. The authors further tested the association between ER and NF-κB. The regulatory involvement of ERs in inflammatory responses was previously shown in different cells, but was not clearly characterized in liver tissues. Interestingly, the authors (Iyer *et al*[6]) demonstrate controversial data that reminds the effects which were detected in colon, gastric, and oesophageal cancers[12-14]. A weak negative correlation was found between nuclear ERα and pNF-κB in normal liver tissues, while a weak positive correlation between nuclear ERα and pNF-κB was detected in the HCV-related HCC group. The change of "polarity" for ER-related signalling confirms the previously suggested dependence of ER effects on different sets of co-factors[15]. The cancer-specific set of co-factors reverses or changes the tendency of ER-mediated biological effects. The kind of co-factors which mediate ER variant signalling in normal and diseased livers remains to be identified in future studies.

Cytoplasmic ER signalling is not well-defined in HCC, either. Iyer *et al*[6] data demonstrated that ERα:ERβ ratio was increased in the cytoplasmic compartment of the HCV-related cirrhosis and HCC groups compared to normal samples. The data indicates increased ERα cytoplasmic presence. However, the analysis of NF-κB activation pathway and cytoplasmic ER subtypes show a strong negative correlation in the HCC group. These findings open wide horizons for future investigations as the range of inflammation and proliferation-related cytoplasmic mediators of ER signalling includes MAPK, PI3K, and the Sphingolipid network. Besides ER-related signalling pathway in cytoplasm, membrane ER should not be neglected in future studies assessing HCC. G protein-coupled estrogen receptor (GPER) is a novel estrogen-binding receptor involved in many pathological conditions, including cancer. The role of the GPER in HCC was recently shown in estrogen-induced protection against HCV[16], but this requires further testing. The availability, quality, and specificity of ER-isoform specific antibodies is being constantly improved, giving hope that the role and mechanism of estrogen/ER signalling in the liver will be clarified in near future.

CONCLUSION

The reviewed data[6] indicated a favorable tendency towards nuclear translocation of ERα and ERβ proteins followed by NF-κB transcriptional activation in HCV

and HCC patient samples. According to this finding, testing ER modulators that target both ERα/ERβ isoforms would be a suggestive clinical strategy. However, ERα (ESR1) mRNA transcriptional levels were decreased in the HCC male liver compared to normal liver samples[6]. Since males are more susceptible to HCC development, the finding of decreased ERα in HCC male samples directs clinical design towards the use of ERβ modulators as a potential anti-HCC therapy. However, the rather low number of tested samples suggests the necessity for more and larger investigations. The change in ER isoform expression ratio and receptor localization indicates the complexity of ER signaling pathway and its transformation during oncogenesis, which we still do not entirely understand.

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REVIEW

Relationship between intestinal microbiota and ulcerative colitis: Mechanisms and clinical application of probiotics and fecal microbiota transplantation

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Abstract

Ulcerative colitis (UC) is an inflammatory disease that mainly affects the colon and rectum. It is believed that genetic factors, host immune system disorders, intestinal microbiota dysbiosis, and environmental factors contribute to the pathogenesis of UC. However, studies on the role of intestinal microbiota in the pathogenesis of UC have been inconclusive. Studies have shown that probiotics improve intestinal mucosa barrier function and immune system function and promote secretion of anti-inflammatory factors, thereby inhibiting the growth of harmful bacteria in the intestine. Fecal microbiota transplantation (FMT) can reduce bowel permeability and thus the severity of disease by increasing the production of short-chain fatty acids, especially butyrate, which help maintain the integrity of the epithelial barrier. FMT can also restore immune dysbiosis by inhibiting Th1 differentiation, activity of T cells, leukocyte adhesion, and production of inflammatory factors. Probiotics and FMT are being increasingly used to treat UC, but their use is controversial because of uncertain efficacy. Here, we briefly review the role of intestinal microbiota in the

pathogenesis and treatment of UC.

Key words: Fecal microbiota transplantation; Intestinal microbiota; Ulcerative colitis; Probiotics; Mechanism; Clinical application

Core tip: As we all know, genetic factors, host immune system disorders, intestinal microbiota dysbiosis, and environmental factors contribute to the pathogenesis of ulcerative colitis (UC). In this review, we explore the mechanism and clinical application of intestinal microbiota such as probiotics and fecal microbiota transplantation in UC so that we can use these tools to cure more diseases. Enteric microbiota leads to new therapeutic strategies for UC.

Shen ZH, Zhu CX, Quan YS, Yang ZY, Wu S, Luo WW, Tan B, Wang XY. Relationship between intestinal microbiota and ulcerative colitis: Mechanisms and clinical application of probiotics and fecal microbiota transplantation. *World J Gastroenterol* 2018; 24(1): 5-14 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i1/5.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i1.5

INTRODUCTION

The etiology and pathogenesis of ulcerative colitis (UC) are complex. UC is believed to be caused by an imbalance between intestinal microbiota and mucosal immunity, resulting in excessive intestinal inflammation[1]. Thus, dysbiosis of intestinal microbiota contributes to the pathogenesis of UC. In UC patients, the intestinal microbial population and functional diversity and stability of intestinal bacteria are impaired, with reductions in specific Firmicutes bacteria and increased Bacteroidetes bacteria and facultative anaerobes[2].

Probiotics are increasingly being used to treat UC. Probiotics are living nonpathogenic bacteria, such as *Lactobacillus, Bifidobacterium,* and *Enterococcus*[3]. The bacteria in probiotics can benefit the intestinal and immune systems[4]. Probiotics can restore the function of disturbed mucosal barrier[5], correct intestinal microbiota imbalance, inhibit competition of potential pathogens, improve local and systemic immunity, and enhance intestinal barrier function[6]. Probiotics can effectively induce and maintain remission in UC patients, suggesting that optimizing intestinal microbiota is key in the treatment of UC.

Fecal microbiota transplantation (FMT) also shows promise in UC. FMT aims to quickly restore the normal function and composition of intestinal microbiota, which can help treat intestinal and parenteral diseases. How FMT improves inflammatory bowel disease (IBD) is not completely understood. Studies have shown that after FMT, the intestinal microbiota composition of recipients was consistent with that of the donors[7]. The mechanisms of action of FMT might be the competitive inhibition of pathogenic microorganisms and improvement of immunity and metabolism.

PATHOGENESIS OF UC

The pathogenesis of UC is complex and it is believed to be mediated by genetic susceptibility, microbial dysregulation, and environmental factors. In UC, mucosal permeability increases and the inflammatory reaction is caused by excessive reaction below the lymphoid tissue[8]. There exists impaired ileum barrier function, the reduction of mucin and goblet cells which produce mucin, and the decrease of epithelial NLRP6 in UC patients[9].

INTESTINAL MICROBIOTA

The intestinal microbiota and the bacteria that compose it, such as Firmicutes, Bacteroidetes, and Actinomycetes, are still not well understood[10,11]. According to aerobiosis or anaerobiosis, the intestinal microorganisms are divided into anaerobic bacteria, facultative anaerobic bacteria, and aerobic bacteria; anaerobic bacteria are the most abundant intestinal bacteria[12]. These bacteria are mainly distributed in the colon and distal small intestine. Most of the bacteria live on the surface of the intestinal mucosa. They attach to the surface of the intestinal epithelial cells to form a layer of bacterial biofilm, which ultimately affects the intestinal metabolism of nutrients, intestinal permeability, and intestinal immune system function.

According to their role in the host, intestinal microbiota can be divided into three categories. The first category contains the physiologic bacteria that are symbiotic with the host. They attach to the deep mucosal epithelial cells, and most are anaerobic bacteria. They are the dominant microbiota of the intestine (*e.g.*, *Bifidobacterium, Bacteroides,* and *Peptococcus*) and play key roles in nutrition and immune regulation. The second category contains conditional pathogens that inhabit the host. They are mainly facultative aerobic bacteria and intestinal nondominant bacteria (*e.g.*, *Enterococcus* and *Enterobacter*). These organisms are harmless when intestinal microecological balance is maintained but can be harmful to humans under certain conditions. The third category contains mostly pathogens (*e.g.*, *Proteus* and *Pseudomonas*). When microecology is in balance, long-term colonization of pathogens is rare, and the number of these organisms is small and nonpathogenic. If changes in the enteral and external environments lead to a decline of intestinal-dominant microbiota, then intestinal microbiota imbalance will occur, with pathogens or conditional pathogens increasing to the point of causing disease.

Intestinal mucosal symbiotic bacteria promote intestinal stability. Symbiotic bacteria help inhibit intestinal colonization by pathogens. Highly evolved bacteria occupy the intestine and prevent pathogens from invading the lamina propria layer. Intestinal bacteria stimulate the intestinal mucosa by causing a low level of inflammation. This inflammation contributes to the formation and improvement of the intestinal immune system, which monitors and removes harmful bacteria to maintain the body health. Intestinal bacteria also participate in important physiologic metabolic activities. *Propionibacterium freudenreichii ET-3* can produce large amounts of vitamin K_2 precursor material, which can activate aroma receptors, participate in substance metabolism, and detoxify and inhibit dextran sodium sulfate-induced colitis *in vivo*[13]. When the composition of intestinal symbiotic bacteria changes, the intestinal microbiota becomes unbalanced, causing an intestinal immune response[14].

Microorganisms release many biologically active substances, such as intestinal short-chain fatty acids (SCFAs), which have a strong immunomodulatory effect. Acetic acid, butyric acid, and propionic acid are the most abundant SCFAs[15]. SCFAs act as the main energy substrates and directly influence the host digestive tract through phenotypic alteration of colonic epithelial cells. They can also act as tumor suppressors and have recently been shown to be modulators of the intestinal neuroendocrine system. In addition, SCFAs are involved in anti-inflammatory gene regulation processes both *in vitro* and *in vivo*[16]. *Faecalibacterium* (*F.*) *prausnitzii* and *Roseburia* are butyric acid-producing bacteria that are usually considered as probiotic microorganisms. Butyrate can protect the integrity of the intestinal epithelium, promote the intestinal immune response, inhibit the growth of tumor cells, and reduce the activity of cancer-promoting enzymes, thus protecting the intestinal wall and reducing intestinal inflammation and colorectal cancer incidence[17].

INTESTINAL MICROBIOTA AND UC

There is close relationship between the pathogenesis of UC and intestinal microbiota. The steady state of the intestinal microbiota is important in preventing the excessive growth of certain microorganisms. Dysbiosis of the intestinal microbiota may be a contributing factor in some diseases and conditions, such as obesity, metabolic syndrome, autoimmune diseases, necrotizing enterocolitis, skin disease, UC, Crohn's disease (CD), and irritable bowel syndrome[18]. When the balance of intestinal microbiota is broken, the intestinal defense function and immunoregulatory function are decreased, the immunity of the body is reduced, and the relative pathogenic factors are increased so as to cause the intestinal mucosal invasion or aggravate the diseases. The pathogenesis of UC is complex, and the interaction between the host and intestinal microbiota may be a key factor. Under normal circumstances, the host's innate and adaptive immunity prevents the invasion of harmful bacteria while tolerating the normal microbiota. However, if the microbiota is imbalanced, immunity is compromised. The intestinal mucosal immune response is overstimulated, which can lead to disease[19].

Disrupted intestinal microbiota can cause intestinal inflammatory responses. Disruption in the microbiota results in a rapid increase in harmful bacteria in the intestine. In addition, release of enterotoxin increases intestinal mucosal permeability, and production of immunosuppressive protein results in immune dysfunction. Growing populations of harmful bacteria directly invade and damage the intestinal epithelial cells, resulting in damage to the intestinal mucosal barrier. Excessive growth of some bacteria affects metabolic and energy metabolism, triggering intestinal inflammation and damage of the intestinal mucosa. Intestinal mucosal barrier function declines, the shield function of the intestinal wall diminishes, and intestinal microbiota is translocated, which further damages the intestinal mucosal barrier, causing a vicious cycle and aggravating the intestinal inflammatory response. Animal experiments have found that colitis can be induced in animal models by disrupting intestinal bacteria. Sterile animals with IL-10 or HLA-B27 knockout did not develop colitis[20], suggesting that intestinal microbiota is essential for the occurrence of UC.

A large number of bacteria are adhered to intestinal epithelial cells of CD patients compared with healthy people. Microbiota might be one of the key factors of activating the intestinal immune system and inducing CD. In addition, the dysregulation of resident microbiota may be the major factor of inducing IBD. The occurrence of intestinal inflammation might cause the production of a variety of different types of instant cell factors.

Microbial pathogens related to UC

Many studies have demonstrated that the composition and function of intestinal microbiota in UC patients are compromised. Some bacteria such as *Akkermansia* (*A.*) *muciniphila* are decreased. *A. muciniphila* is one of the most abundant members of the human gut microbiota, representing between 1% and 5% of human intestinal microbes. Several studies have shown a relationship between UC and *A. muciniphila*. *A. muciniphila* was decreased in UC patients, along with *Roseburia* bacteria[21]. It was also decreased in a mouse model of carrageenan-induced colitis[22]. Therefore, *A. muciniphila* might be a new target of UC. Abnormal microbiota reduced the complexity of the intestinal microbial ecosystem, which is a common feature of patients with UC and CD[23]. Several microbial pathogens are possibly related to intestinal inflammation and thus UC patients

may harbor *Mycobacterium avium paratuberculosis*[24,25], adherent-invasive *Escherichia coli*[26], *Clostridium* (*C.*) *difficile*[27-29], *Helicobacter* species[30-32], *Salmonella* species[33], *Yersinia* species[34], *Fusobacterium* species[35], norovirus[36], and *Listeria* species[37]. However, the exact pathogenesis is unclear.

SCFA production

In one study, qualitative and quantitative changes were demonstrated in the composition of enteric bacteria in patients with UC[38]. The diversity of bacteria in UC patients was less than that of the control group. The number of dominant bacteria was decreased, the number of pathogens was increased, and the proportions of each strain of the dominant microbiota were not balanced. Studies have found that the diversity of intestinal microbiota in UC patients is decreased by approximately 25% compared with that of healthy controls. *Firmicutes* and *Bacteroidetes* were decreased, while Proteobacteria and Actinomycetes were increased. Machiels *et al*[39] found that two important butyrate-producing Firmicutes bacteria, *Roseburia hominis* and *F. prausnitzii,* were significantly decreased in UC patients. Varela *et al*[40] found that the number of *F. prausnitzii* was significantly increased in the remission period of UC, indicating that *F. prausnitzii* may play a vital role in the treatment of UC.

T-cell response

Some bacteria affect the differentiation of T-cell subsets and thus influence the occurrence and development of inflammation. *Bacteroides* (*B.*) *fragilis* and capsular lipopolysaccharide A improved the balance of Th1/Th2 cells in mice by activating NF-κB *via* TLR2 and regulating the secretion of TNF-α and IL-12[41]. Other studies have found that *B. fragilis* affected the steady state of mucosal T cells by inducing the production of regulatory T (Treg) cells in a 2,4,6-trinitrobenzene sulfonic acid-induced colitis mouse model[42]. In the small intestine and colon, segmented filamentous bacteria (SFB) can increase the number of Treg cells[43] and can also regulate the differentiation of Th17 cells and promote the production of IL-22-producing CD4+ T cells. The mechanism might be that SFB stimulates the production of serum amyloid A (SAA), which is an early inflammatory marker, and SAA induces a specific Th17 response *in vivo*[44]. These findings suggest that lipopolysaccharide A may play a dual role in intestinal homeostasis. Mice treated with *Lactobacillus reuteri* had a higher percentage of Treg cells[45]. *Clostridium* clusters Ⅳ, XIVa, and XVIII secrete SCFAs to stimulate the production of TGF-β by intestinal epithelial cells and induce Treg cell differentiation to promote intestinal mucosal immune tolerance[46]. Hsp65-producing *Lactococcus lactis* inhibited experimental autoimmune encephalomyelitis, and the mechanism might be related to the induction of CD4+/Foxp3+ and CD4+/LAP+ Treg cells and the TLR2 signaling pathway *in vivo*[47] (Figure 1).

MECHANISM AND CLINICAL USE OF PROBIOTICS

Probiotics can promote the secretion of anti-inflammatory factors by improving intestinal mucosal barrier and immune system function and thus inhibiting the growth of harmful bacteria in the intestine. Pathogens can weaken the barrier function of the intestinal mucosa and pass through or damage the mucosal wall. Probiotics can prevent or repair such damage.

The main mechanisms of probiotics include competing for adhesion sites and nutrients, maintaining the balance of normal intestinal microbiota, enhancing intestinal mucosal barrier function, promoting immune tolerance of the intestinal mucosa, interfering with intestinal inflammatory response, and inhibiting intestinal epithelial cell apoptosis. Studies have indicated that probiotics might be useful in the treatment of UC. Through the PI3K/Akt and NF-κB signaling pathway, probiotics decreased proinflammatory cytokines such as TNF-α and IL-1β and increased anti-inflammatory factor IL-10, thus improving the symptoms of UC.

Bifidobacterium is one of the first colonizers of the baby intestine and the main microbial organism of the adult intestine[48]. It is beneficial microbiota that promotes antitumor immunity and prevents recurrence of UC. *Bifidobacterium breve* reduces the shedding of apoptotic epithelial cells in an extracellular polysaccharides- and MyD88-dependent manner[49].

A meta-analysis containing a total of 1763 cases in 32 clinical trials showed that probiotics significantly increased the clinical remission rate of patients with active UC (P = 0.01; risk ratio = 1.51). Probiotics and aminosalicylic acid had similar clinical effects. Subgroup analysis showed that VSL#3 (Alfasigma) resulted in the most significant improvement in UC, followed by lactic acid bacteria and *E coli*[50]. Another study on UC patients aged 12 to 16 years showed that the recurrence rate was lower in the group treated with probiotics for 1 year than in the control group (21% *vs* 73%)[51]. One study showed that the addition of *Lactobacillus* species to the usual therapies reduced relapse in UC patients[52]. Oliva *et al*[53] also found that local administration of *L. reuteri* ATCC 5573 with a standard dose of mesalazine reduced inflammation of the rectal mucosa in children with UC. In addition, *E. coli* strain Nissle promoted long-term maintenance of remission in UC patients. Administration of *Saccharomyces boulardii* during treatment with mesalazine induced clinical remission in 71% of patients with active mild to moderate UC[54,55]. In 2013, Varela and colleagues[40] investigated the fecal bacteria of 116 UC patients in remission and 16 healthy subjects and found that *F. prausnitzii* colonization was

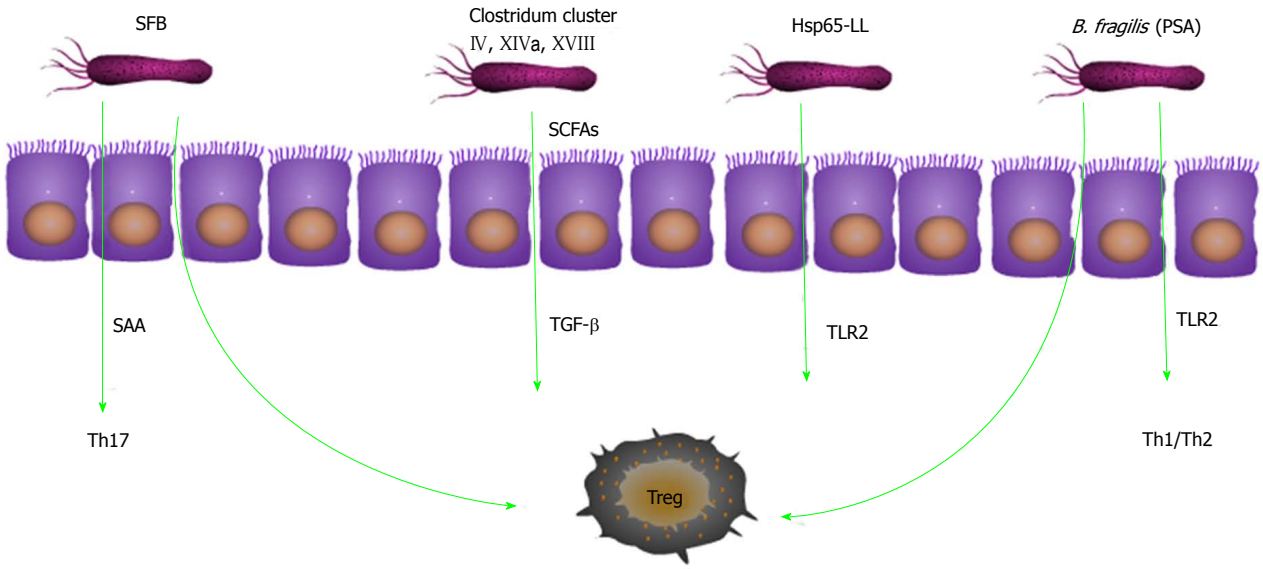

Figure 1 Bacteria affect the differentiation of T-cell subsets and thus influence the occurrence of inflammation. Different types of bacteria have different effects on T cell differentiation. SFB has an effect on TH17. *Clostridum* clusters Ⅳ, XIVa, and XVIII and Hsp65-LL can influence the differentiation of Treg cells. *B. fragilis* might affect the ratio of Th1/Th2 *via* TLR2. SFB: Segmented filamentous bacteria; Hsp65-LL: Hsp65-producing *Lactococcus lactis*; *B. fragilis*: *Bacteroides fragilis*.

significantly reduced in UC patients. In addition, oral administration of *F. prausnitzii* was found to help induce remission in UC patients[56].

However, the results with probiotics in UC have not been consistent. Studies have shown that probiotics can increase gastrointestinal peristalsis, induce diarrhea in IBD patients, change stool frequency, and increase disease activity[57]. A study by Wildt *et al*[58] demonstrated that there was no difference in UC remission between patients administered with *Lactobacillus acidophilus* LA-5 and *Bifidobacterium* and the control group. Larger sample sizes and more randomized controlled clinical trials are needed to clarify the role of probiotics in the treatment of UC.

MECHANISM AND CLINICAL USE OF FMT

Along with probiotics, FMT is also being investigated as a treatment for UC. FMT is the process of transplanting fecal bacteria from a donor into a recipient. Currently, no standard criteria exist for FMT donor screening[59]. Donors are often selected from relatives, spouses, friends, or healthy volunteers. The collection of feces is usually conducted on the day of transplantation. The feces are dissolved in saline or water, homogenized, and filtered to form a homogeneous solution. Stools are usually transplanted in 6 to 8 h[60]. Stool banks exist in many countries and may serve many clinical functions in the future[61,62]; however, internationally standardized stool banks have not yet been established.

The essence of FMT is to reconstruct intestinal microbiota and cure disease by normalizing abnormal immune and inflammatory responses, the numbers and activities of neurotransmitters and vasoactive substances, and energy metabolism. FMT is used to treat chronic gastrointestinal infections and IBD[60]. FMT can improve intestinal microecology and the permeability of the intestinal mucosa. It can activate the intestinal humoral immune response to induce synthesis of IgA, IgG, and IgM through the TLR pathway, thus protecting the intestinal mucosa. FMT can also reduce the pH value of the intestine and increase the adhesion of bacteria and H_2O_2 to competitively inhibit the adhesion and translocation of pathogens[63]. Finally, FMT can treat immune disorders by inhibiting the secretion of proinflammatory cytokines and promoting Th1 differentiation, T-cell activity, leukocyte adhesion, and immune-stimulatory factors.

In gastrointestinal homeostasis, the diversity of the microbiota prevents colonization and overgrowth of pathogens. FMT can reduce intestinal permeability by increasing the production of SCFAs, thereby reducing the severity of disease. Increased SCFAs, especially butyrate, which is the main source of energy in colonic epithelial cells, maintain the integrity of the epithelial barrier by reducing intestinal permeability. FMT can also restore the dysbiosis of microbiota. The proportions of beneficial bacteria are increased and the diversity is also increased. FMT can make the composition of microbiota be more likely to be similar to the donor for a long time. Recently, a study by Dutta *et al*[64] found that FMT increased the diversity of fecal microbiota and increased the proportion of Lachnospiraceae, which are butyrate-producing bacteria. These findings not only confirm the speculated mechanism of action of FMT, but also indicate that Lachnospiraceae might be the key bacteria in the success of FMT. This finding could lead to targeted and standardized FMT.

FMT is successful in treating recurrent *C. difficile*

Table 1 Main case series and reports of fecal microbiota transplantation in ulcerative colitis patients

Ref.	UC (*n*)	Stool material	Volume infusion	Infusion route	Frequency	Donor relationship	Characteristics of outcomes
Kump *et al*[92], 2013	15	Fresh	100 to 150 g 300-500 mL	Rectosigmoidoscopy	1	Unrelated	None of the patients achieved CR
Kunde *et al*[75], 2013	10	Fresh	70 to 130 g 60 mL	Enemas	5	Related	33% patients achieved CR at 1 wk
Angelberger *et al*[72], 2013	5	Fresh	60 g 250 mL	Nasojejunal tube	5	Related or unrelated	(0/5) patients demonstrated a remission
Suskind *et al*[93], 2015	4	NR	30 g 100 mL	Nasogastric Tube	1	Related or unrelated	(0/4) patients demonstrated a remission
Kellermayer *et al*[86], 2015	3	Frozen	NR	Colonoscopy	22-30	A single donor	(3/3) patients demonstrated a remission
Damman *et al*[94], 2015	7	Fresh	175-290 mL	Colonoscopy	1	Related or unrelated	(1/7) patients demonstrated a temporary remission
Wei *et al*[67], 2015	11	Fresh	60 g 300 mL	Colonoscopy	5	Unrelated	The Mayo scores of all patients decreased at 4 wk
Cui *et al*[66], 2015	15	NR	NR	Gastroscope channel	1	1 (73.3%) related; 2 (26.7%) unrelated	28.6% patients achieved CR
Vermeire *et al*[95], 2016	8	Fresh	100 g 200 mL	Nasojejunal tube or colonoscopy	2	Related	(2/8) demonstrated a remission at week 8

UC: Ulcerative colitis.

Table 2 Recent randomized, controlled trials of fecal microbiota transplantation in ulcerative colitis patients

Ref.	Rossen *et al*[69], 2015	Moayyedi *et al*[70], 2015	Paramsothy *et al*[96], 2017
n (UC/placebo)	48 (23/25)	75 (38/37)	85 (42/43)
UC arm	50 mL, nasoduodenal, healthy donors	50 mL enema, healthy donors	150 mL, colonoscopic, unrelated donors
Placebo arm	Autologous FMT	50 mL enema, water	150 mL, colonoscopic, isotonic saline
Frequency	At weeks 0 and 3	Once weekly for 6 wk	5 d per week for 8 wk
Evaluation criterion	Remission (SCCAI $\leqslant$ 2 + $\geqslant$ 1-point decrease in the Mayo endoscopic score) at week 12	Remission (a Mayo score $\leqslant$ 2 with an endoscopic Mayo score of 0) at week 7	Remission (Mayo score $\leqslant$ 2, all subscores $\leqslant$ 1, and $\geqslant$ 1 point reduction in endoscopic subscore) at week 8.
Results	30% with FMT *vs* 20% controls (P = 0.51)	24% with FMT *vs* 5% placebo (P = 0.03)	27% with FMT *vs* 8% placebo (P = 0.021)

FMT: Fecal microbiota transplantation; UC: Ulcerative colitis.

infection. FMT could also potentially be used to treat UC, although limited evidence exists. Only a few small case series have been reported, and the efficacy is inconsistent, ranging from 20% to 92% (Table 1). There are currently only three randomized controlled trials of FMT in UC (Table 2). Clinical trials have shown differences in efficacy of FMT, which might be due to differences in individual factors, severity of disease, sites of disease, FMT donors, infusion paths, infusion doses, and so on. A recently reported meta-analysis[65] included 11 studies (containing two randomized controlled trials, one open case-control study, and eight cohort studies) and 133 patients with UC. The results showed that the clinical remission rate was 30.4% in UC patients treated with FMT. There were no significant differences in the remission rates between single and multiple transplantations and between upper digestive tract and lower digestive tract transplantations. In contrast, some reports have indicated that repeated FMT may be better than single transplantation[66]. Although the remission rate of UC was not ideal, patients' quality of life improved[67], and FMT is easy to perform and safe. Another meta-analysis of FMT showed that the clinical response rate of IBD was 22%[68]. After FMT, the intestinal microbiota diversity of UC patients increased, and the microbiota species converged with those of the donors[69-71]. However, in time, intestinal bacteria will revert back to the pretreatment state. Therefore, repeated FMT may be needed; thus, the ideal number of treatments and interval between transplantations need to be explored[72].

ADVERSE REACTIONS OF INTESTINAL MICROBIOTA

Few adverse reactions have been reported with probiotics[73,74], and FMT has also been regarded as a safe treatment strategy. Adverse events or serious complications of FMT are rarely reported but include abdominal cramps, bloating, constipation, and diarrhea. Fever and increased C-reactive protein level are common, but both are transient and self-limiting[75]. Other rare adverse reactions include sore throat and headache[76], abnormally low blood pressure[77], shingles[78], the occurrence of UC[79], norovirus infection[76], weight gain[80], peritonitis or enteritis[81,82], perforation of the colon, pneumonia[83], and cytomegalovirus infection[84]. Bacteremia has been reported after FMT[85]. After FMT, changes in the composition of intestinal microbiota

may lead to changes in gene expression of the mucosal cells of the recipients[86], alteration of intestinal mucosal immune function[87], changes in the intestinal ecological environment[88], and differences in body metabolism[89]. FMT can affect gastrointestinal diseases as well as extra-gastrointestinal disorders[90]. Therefore, patients who undergo FMT require long-term follow-up to observe pathologic and physiologic effects of treatment.

In addition, because of the diversity and uncertainty of intestinal microbiota, the current treatment criteria for FMT are difficult to formulate. Controversy concerns whether intestinal microbiota should be managed as a drug or as a bodily organ. These problems hamper the standardization and specific application of FMT. Determining the effective bacteria and the ideal treatment subjects will help to standardize FMT and make treatment more consistent[91].

CONCLUSION

The pathogenesis of UC is closely related to intestinal microbiota, although the exact bacteria that contribute to UC have not been determined. The use of probiotics in UC is currently being investigated. Probiotics may help normalize the imbalance of intestinal microbiota, improve the microecological environment, enhance intestinal mucosal barrier function, and reduce gastrointestinal infections. However, determination of the specific mechanism and types of probiotics, the best concentrations, and ideal treatment methods requires further study. The treatment of UC with FMT is also promising, but many questions remain regarding this treatment as well. Clearly, more research is needed regarding the use of probiotics and FMT in UC.

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MINIREVIEWS

Updated review on immune factors in pathogenesis of Crohn's disease

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Abstract

Although the incidence of Crohn's disease (CD) in China is not as high as that in European and American countries, there has been a clear increasing trend in recent years. Little is known about its pathogenesis, cause of deferment, and the range of complications associated with the disease. Local and international scholars have presented many hypotheses about CD pathogenesis based on experimental and clinical studies, including genetic susceptibility, immune function defects, intestinal microflora disorders, delayed hypersensitivity, and food antigen stimulation. However, the specific mechanism leading to this immune imbalance, which causes persistent intestinal mucosal damage, and the source of the inflammatory cascade reaction are still unclear. So far, the results of research studies differ locally and internationally. This paper presents the most current research on immune factors in the pathogenesis of CD.

Key words: Pathogenesis; Immune; Immunotherapy; Intestinal inflammation; Crohn's disease; Cytokines; Lymphocytes; T lymphocytes

Core tip: It is now clear that Crohn's disease (CD) is an autoimmune disease that involves at least the intestinal mucosal immune system, when the mucosal immune system is invaded by food or bacterial antigens. However, it is worth mentioning that the mechanism remains unclear. Whether activation of the immune system is the internal defects (constitutive activation or regulation mechanism disorder) or changes in the epithelial mucosal

barrier leading to continuous stimulation is still not clear. The mechanism of CD is intensively studied by domestic and foreign scholars on the immune destruction. Here we summarize the latest research status on immune factors in the pathogenesis of CD with regard to the mechanisms about T-lymphocyte immunity, innate lymphocyte immunity, cytokines and immune therapy.

Li N, Shi RH. Updated review on immune factors in pathogenesis of Crohn's disease. *World J Gastroenterol* 2018; 24(1): 15-22 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i1/15.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i1.15

INTRODUCTION

Crohn's disease (CD) is a chronic granulomatous inflammatory disorder that can involve any part of the gastrointestinal tract, predominantly the terminal ileum and adjacent colon, and presents with segmental, asymmetric distribution of granulomatous inflammation[1]. The main clinical symptoms are abdominal pain, diarrhea, fistula, anal lesions, and systemic symptoms of different severity in the body. The incidence and prevalence rates of CD have been increasing rapidly. Zheng *et al*[2] analyzed the current status and prevalence changes of CD in mainland China in recent decades, and found that the CD incidence and prevalence rates in the last decade were 1.21 per 100000 persons/year and 2.29 per 100000 persons, respectively. These rates are higher than those during 1950-2002, 0.28 per 100000 persons/years and 1.38 per 100000 persons, respectively[2]. However, the pathogenesis, cause of deferment, and variety of complications are not clear[3].

Scholars have presented many hypotheses about the pathogenesis of CD. Some have suggested that the environment is filled with intestinal pathogens or opportunistic pathogens, such as pathogenic *Escherichia coli*, which spread through patients' intestinal epithelial cells, and if innate immune cells such as monocytes, neutrophils, and natural killer (NK) cells cannot kill these translocated bacteria, they function as antigens and keep stimulating intestinal mucosal cells to cause immune responses[4], like abnormal Th1 and Th17 activation. Th17 activation results in the formation of granulomas[5]. Some antigens produced by bacteria can induce CD4+ T cells to differentiate into targeted cytotoxic T lymphocytes (CTLs), and these cells release interleukin (IL)-17 to stimulate Th17 cells to produce transforming growth factor and interferon-alpha (IFN-α), which can cause persistent inflammation and fibrosis[6]. Another opinion presented by Papadakis is that after the early antigen enters the body, associated lymphoid tissues are stimulated. When this occurs, the body becomes sensitive to the antigen, creating a sensitive state and mucosal immunity for the antigen of normal intestinal bacteria, and from then on, any secondary damage to the intestinal mucosa barrier results in the antigen contacting the lymphoid tissue again, stimulating a severe local immune response[7,8]. However, intestinal mucosa barrier damage occurs all the time, yet, most often, there is no immune response. This obviously means that this disease is also related to the body's genetic susceptibility and environmental factors[9].

These theories have some differences related to gene mutations causing immune dysfunction, delayed hypersensitivity, alterations of intestinal flora, and activation induced by some special food[10] or bacterial antigen. However, each mechanism ultimately involves the immune response and broken immune tolerance. Immunity is now a universally acknowledged cause of CD, and hormone therapy can cause remission, demonstrating that CD is an immune-related disease. At present, the specific processes involved in the breakdown of immune tolerance and the associated sequence of genetic changes are still unclear[11-13]. Also, the specific mechanisms leading to this immune imbalance, which causes persistent intestinal mucosal damage, and the source of the inflammatory cascade reaction remain unclear. So far, the results of research studies differ locally and internationally. Here we present the most current research on immune factors in the pathogenesis of CD with regard to the mechanisms about T cell immunity, innate cell immunity, and cytokines.

CELL IMMUNITY AND INFLAMMATORY FACTORS

T lymphocytes

CD4+/CD8+ T lymphocytes and cytokines (Figures 1 and 2): Some studies have found that CD results mainly from chronic inflammation of T lymphocytes, especially CD4+ T cells. As cells that mediate humoral immunity, B lymphocytes do not participate in the occurrence and development of CD. CD4+ T cells are the main effector lymphocytes in intestinal inflammatory tissue. Eventually, despite the differences in the development of the inflammatory process, the process of inflammation is induced by Th1 or Th2 cells. The two subgroups of cytokines produced by Th1 or Th2 cells are mutual antagonists. Once a group of cytokines are produced earlier or more than the other, they will inhibit the other group of cytokines. Therefore, for the same inflammation, the two kinds of CD4+ T cells can lead to different consequences, presenting two different types of immune response, CD and ulcerative colitis (UC). Lymphocytes play a dominant role in CD patients, mainly secreting IL-12, IFN-α, tumor necrosis factor (TNF)-γ, IL-1, IFN-γ, IL-2, and other cytokines[14-16]. But for patients with UC in the intestinal mucosa, the characteristics of Th2 lymphocytes are atypical infiltration and production

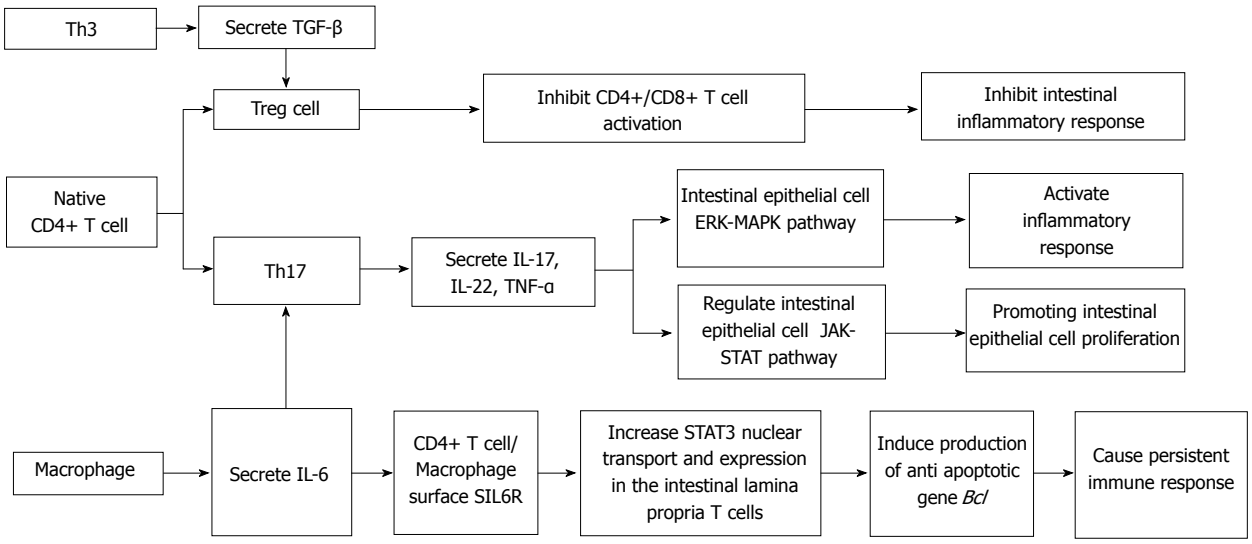

Figure 1 The main immune process of Crohn's disease.

of IL-4, IL-5, 1L-13, and transforming growth factor (TGF)-β cytokines. Therefore, more autoantibodies are present in UC patients than in CD patients, mainly Th2 type immune response related antibodies. In CD animal models, intestinal bacteria and some specific antigens, such as certain pathogenic *Escherichia coli*, can induce intestinal mucosal Th1, which induces a cellular immune response. However, cellular immunity can be induced in a specific susceptible animal model such as *IL-10* gene knockout mice, whereas cellular immunity cannot be induced in wild type mice[17-19]. This also suggests that gene deletion and mutation cause the natural immunity defects that induce CD. However, there is growing evidence that the Th1-Th2 classification is too simple; the two paths of mutual exclusion hypothesis has been questioned, and increasing evidence shows that IL-4 and IL-13 from the Th2 cells take part in ileal CD. In CD, there are two simultaneous changes in the initial stage of inflammation: the induction phase and the effector phase. Both Th1 and Th2 may participate in each phase simultaneously or sequentially.

Studies have found that CD8+ cells also exist in the intestinal mucosa. For example, many CTLs are observed in CD models, indicating a greater effect than in other diseases. When a gene related to the function of CD8+ T cells is deleted, there is no influence on CD inflammation, whereas when genes related to the function of CD4+ T cells are deleted, there is an improvement in intestinal mucosal inflammation. Therefore, the inflammatory response appears to be related to CD4+ T cells, and the role of CD8+ T cells in CD remains unclear. However, the pathological effect of CD8+ cells should not be ruled out completely[19,20]. So far, the role of CTLs in the pathogenesis of CD has been investigated in few studies, but CD8+ cell subsets and T lymphocytes (CTLs, CD8, and CD28+) are believed to play a key role in the recognition and elimination of mutant cells, and inflammation caused by graft-versus-host reactions. Compared with healthy subjects, CTLs in CD patients release enzymes, such as perforin and telomerase, and increase cytotoxic protein activity. Studies have compared the chromosomal changes of CD8+ cells in patients with CD to those of normal patients. Early activation of CD8+ cells may determine subsequent proliferation, cytokine production, and antigen recognition capability. Thus, an intervention causing early activation of CD8 cells is a potential therapeutic option[21]. It has been found that the K+ pathway plays an important role in the activation of T cells in the early stage of CD, and in the expression of Kv1.3 and IKCa1. Additionally, many scholars have reported two kinds of potassium ion channel that play an important role in mouse and human inflammatory bowel disease (IBD) development. The basic function of these ion channels is to maintain intracellular negative potential so that the influx of calcium in the body fluids activates the immune function[22]. These studies have shown that the effects of CD8+ cell toxicity and T cell depletion on CD inflammation are different, but different Th1 mediated inflammatory responses play a major role in the pathogenesis of CD. Therefore, the immune balance is deranged, and persistent inflammatory response occurs.

New T lymphocyte subsets and cytokines (Figures 1 and 2)

In recent years, researchers have found a new subset of T cells, called T regulatory (Treg) lymphocytes. When human or mouse CD4+ T cells were exposed to repeated stimulation with IL-10 *in vitro*, a new subset of T cells that secrete a high level of IL-10 but a low level of IL-2 was induced. Treg cells have weak proliferation ability[23], but their cytokine secretion pattern is quite stable. They can secrete a small amount of TGF-β, inhibit antigen-specific immune responses, and restrict intestinal inflammation through down-regulation of IL-10[24]. Treg cells are a group of T

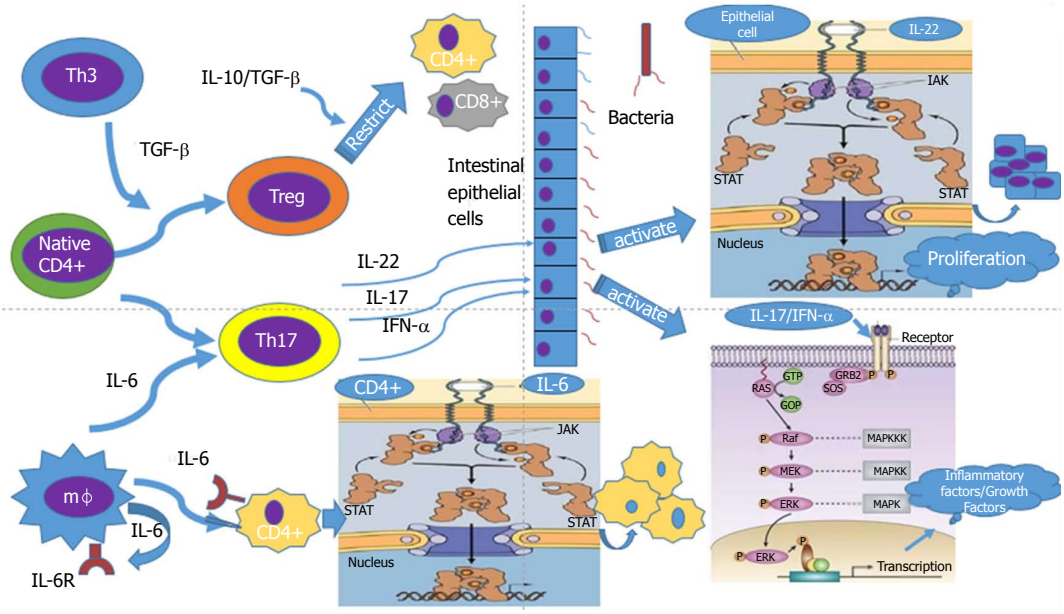

Figure 2 The main immune mechanisms of Crohn's disease.

cell subsets with immunosuppressive effects. They can negatively regulate the immune system by inhibiting the proliferation and activation of CD4+ and CD8+ T cells. Th17 cells are a newly discovered helper T cell subset, named after its secreted cytokine IL-17. Th17 cells play important roles in innate immunity and acquired immunity by secreting IL-17A/F, IL-22, and IL-21. TNF-α is an inflammatory cytokine in host defense against extracellular bacterial infection, in anti-parasite immunity mediated by chronic inflammation, and in organ transplant rejection. Th17 cells have received extensive attention in many important physiological or pathological processes in immune diseases and cancer, especially in the study of the pathogenesis of autoimmune diseases[25]. Researchers have found that Th17 and Treg cells are subsets of CD4+ T cells. Th17 cells promote intestinal inflammation induced by autoimmune diseases, while Treg cells inhibit intestinal mucosal inflammation, which means that they have opposing functions. A recent study found that TGF-β can induce naive T cells into Treg immunosuppression. In the presence of IL-6, TGF-β promotes the differentiation of naive T lymphocytes to Th17 cells, which secrete proinflammatory cytokine IL-17, and the occurrence of autoimmunity and inflammation[26]. Treg cells can repair mucosal inflammation in patients with IBD, but under the influence of IL-6 andor IL-23, Treg cells differentiate into Th17 cells, which secrete large amounts of IL-17[27]. Kinugasa pointed out that IL-17 participates in the regulation of intestinal epithelial barrier function through the extracellular signal regulated (ERK)-mitogen activated protein kinase (MAPK) pathway, which may be a potential cause of intestinal inflammation[28].

Carrier *et al*[29] reported another kind of T cell that mediates oral tolerance by TGF-β, which is called Th3 cells. The difference between Th3 and Treg cells is mainly that the former secretes high levels of TGF-β, inhibiting immune responses. Due to the lack of an immunomodulatory effect, *IL-10* or *TGF-α* gene knockout mice easily suffer from autoimmune colitis. Therefore, it is speculated that Treg and Th3 cell dysfunction is involved in the pathogenesis of IBD, especially CD. Because previous studies of Th1 lymphocytes cannot fully explain the pathogenesis of CD, new T cell types such as Th17, Treg, and Th3 have become the mainstream of the IBD system, which provides a new direction for the treatment and in-depth study of CD.

Intestinal lamina propria innate lymphocytes and cytokines

NK cells are a lymphoid cell lineage that is considered a part of the natural immune system. Innate immunity is a natural immune defense function formed through the body's process of evolution and development. However, since the discovery of lymphoid tissue induction (LTi) cells in 1997, the lymphocyte populations in the innate immune system have been continuously expanded[30]. The loss of LTi cells leads to ROR γt expression defects, and the mice could not form lymph nodes and isolated lymphoid follicles, which means that LTi cells are crucial for lymphatic tissues and organs, and the latter is the basis of the intestinal mucosal immune barrier and intestinal immune homeostasis. In recent years, researchers have found some new immune cells in the mouse and human mucosal tissue. These cells belong to the lymph cell lineage in the form and development degree, but do not express specific antigen recognition receptors on the surface of the

mature lymphocytes. They are similar to NK cells. Due to the characteristics of LTi cells, they are called innate lymphoid cells (ILCs)[31]. Numerous studies have shown that ILCs participate in the regulation of intestinal mucosa homeostasis and play an important role in the pathogenesis of IBD through multiple pathways. Based on secreted factors and the transcription factor expression pattern of different cells, the family of ILCs is divided into three categories: (1) ILC1, including classic NK cells, tissue residing NK cells, and mucosal ILC1, which express T box transcription factor (T bet) and/or Eomes. Under the stimulus of IL-12 and IL-18, ILC1 produce IFN-γ, which is mainly responsible for antiviral, bacterial, and toxoplasma infections and also plays a role in the immune memory; (2) ILC2, including natural helper (NH) cells, which produce Th2 cytokines, such as IL-5 and IL-13, and play an important role in parasite infection, allergy, and asthma; and (3) ILC3, including all the ILC subsets that produce IL-17 or IL-22, with LTi being the earliest reported ILC subset, which can induce intestinal lamina propria isolated lymphoid follicles and the formation of lymphoid tissue by expressing the ROR-γ transcription factor[32,33]. A study[34] using model mice infected with *Citrobacter* and *Candida albicans* showed that ILC3 secrete IL17 and IL22, which can promote Paneth cells to secrete antimicrobial peptides (such as Reg Ⅲβ and Reg Ⅲγ). These peptides can block the contact between bacteria and epithelial cells and inhibit intestinal inflammation. Some studies have shown that the reduction of IL-22 + ILC3 in the intestinal mucosa is associated with the occurrence of IBD[35]. IL-22 promotes epithelial cell proliferation through the JAK-STAT pathway, thereby preserving the integrity of the epithelial barrier and hindering intestinal microbial invasion[36]. The deficiency of IL-22 causes damage to the intestinal mucosal barrier, which leads to the exposure of intestinal tissue to many antigens and induces an abnormal immune response in the genetic susceptible host to cause the occurrence of IBD[37]. Meanwhile, macrophages and the intestinal secretion of IL-1β can stimulate ILC3 to produce granulocyte macrophage colony stimulating factor (GM-CSF), which plays a role in T cell proliferation[38]. Inhibition of GM-CSF can lead to a decrease in the number of regulatory T cells and immune tolerance defect, which also plays a role in the occurrence and development of IBD.

Studies have found that increased ILC3 can overexpress major histocompatibility complex (MHC) II[39,40]. ILC3 as antigen-presenting cells can, *via* MHCII, induce CD4+ T cell apoptosis, and thus avoid the T cell response to produce intolerance to intestinal symbiotic bacteria. Further studies of IBD patients compared with non-IBD patients found that the expression of MHCII by ILC3, which can induce CD4+ T cell apoptosis, was significantly reduced in IBD patients, thus causing an immune reaction against commensal bacteria and damaging the intestinal mucosa. Therefore, the intestinal mucosa barrier damage and immune tolerance defects cause a disorder in intestinal homeostasis, which plays an important role in the pathogenesis of IBD, the maintenance of homeostasis of the body, and the coordination among the different subtypes that work together. Regarding CD, an increasing number of studies have confirmed that the imbalance in the regulation of ILC breaks down the intestinal tolerance to food and bacterial antigens in the gut leading to CD, with ILC3 being the most important[29,34,36,41]. A study found that the deletion of IL-22+ ILC3 caused the spread of *Alcaligenes sp.* in the intestinal lymph tissue and induced a systemic immune response, which may be closely related to the occurrence of CD[42]. Mast cells stimulate fibroblast proliferation and collagen synthesis, and promote collagen protein aggregation activity and expression of c-kit receptor at the same time. Mast cells also stimulate chemotaxis through stem cell factor receptor (c-kit), and stimulate the proliferation of intestinal cells and fibroblasts[43]. In a previous study, a *Salmonella typhi* strain was used to induce C57/B16 mice to produce severe and persistent bowel wall fibrosis. In this animal model, chronic infection was caused by intestinal flora; large extracellular matrix (ECM) accumulation was seen in the ileocecal region and colon, and fibrosis and extensive transmural inflammation extended to the colon[44], but the most serious and extensive fibrosis was found in the ileocecal valve[45]. A study showed that ILC3 caused overexpression of IL-22, which activates monocytes, macrophages, and mast cells during inflammatory mucosal repair. Finally, the innate lymphocytes in the tissue result in fibrosis. Many studies on inflammatory factors have revealed that IL-22 arguably secreted by ILC3 can promote Paneth cells to secrete antimicrobial peptides (*e.g.*, Reg Ⅲβ and Reg Ⅲγ). These peptides can restrict bacterial contact with epithelial cells and inhibit intestinal inflammation at the same time, and IL-22 promotes epithelial cell proliferation *via* the JAK-STAT pathway to maintain the integrity of the epithelial barrier and to prevent intestinal microbial invasion. Moreover, deletion of IL-22 causes intestinal mucosa barrier damage, and the intestinal tissue is exposed to many antigens, thus inducing abnormal genetic susceptibility to host immune response and leading to CD[34,36,37]. Scholars believe that Th17 cells, *via* inflammatory cytokines such as IL-17, IL-22, IL-21, and TNF-α, are involved in the body's defense of extracellular bacterial infection and parasitic immune resistance, and mediate chronic inflammation, but excessive expression of inflammatory markers leads to CD[25]. Since the results of this study were inconsistent, further studies examining the role of IL-22 in the pathogenesis of CD are needed.

IMMUNOTHERAPY

The traditional view is that CD is due to acquired

immune system disorders, and Th1 cytokines are the main factors in the development of CD. However, only some of them can be used in the treatment of CD, including IL-1, 2, 6, 12, 18, IFN-α, and TNF-γ. They can act on intestinal epithelial cells and cause epithelial cell apoptosis by activating lymphocytes. So far, the most successful example is the use of anti-TNF-α antibodies for the treatment of refractory CD with fistula. Mannon *et al*[46] reported the safety of subcutaneous anti-IL-12 monoclonal antibody for 7 wk, and despite the small number of cases studied, the clinical response was significant when a higher dose of IL-12 monoclonal antibody therapy (3 mg/kg body weight) was given. Long-term safety is a particularly important problem because the Th1 cytokines are important anti-infective factors. For example, long-term inhibition of the host response to tuberculosis (TB) by the treatment of anti-TNF-alpha antibodies may lead to the recurrence of latent TB in the patient; anti-IL-12 treatment should be considered for the possibility of recurrent asthma. Moreover, the etiology of CD is complex, especially in multiple stage disease. In some cases, the cytokines are likely to be harmful rather than beneficial. In some patients, anti-TNF-α antibody (infliximab) treatment does not reduce CD progression, which shows that it is not only due to the Th1 pathway but also due to different mechanisms in the different periods of disease progression. However, the innate immune system is also important, especially in the stage of disease induction; the congenital immune system cells is an important generator of cytokines, such as IL-1, TNF-α, IL-6, and other cytokines, and these factors play an important role in intestinal mucosal inflammation. The CD susceptibility genes encoding intracellular proteins (such as NOD2/CARD15) were found first in the innate immune system, which can activate certain cytokines dependent on NF-kappa B to cause intestinal bacteria to break the immune tolerance of the intestinal mucosa[47]. By using monoclonal antibodies against cell factors, fusion proteins, and receptor antagonists for cytokine blocking, immunomodulation of CD can be done effectively.

CONCLUSION

Research on the relationship between the immune regulation and the pathogenesis of CD has greatly improved the understanding of the role of immune factors in the development of CD. Since the discovery of new types of Th1 cells such as Treg, Th3, and Th17, as well as the application of antibodies to inflammatory factors TNF-α, IL-12, IL-22, and other new cytokines, it is clear that CD is a complex disease, not only due to the Th1 pathway, but also due to different mechanisms in different periods of disease progression, which is why the use of IL-12 or TNF-α antibody treatment in many patients with CD is not so effective. Hereditary susceptibility, the surrounding environment, intestinal flora, and other factors may also be involved. Although there are a variety of cytokine antagonists in clinical application, the results are not satisfactory. So far, we have not found the main immune response pathway involved in CD. So, it is necessary to carry out a large-scale screening of important inflammatory cytokines and inflammatory cells in Asian populations, to find the main cause of the CD immune response pathway.

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ORIGINAL ARTICLE

Basic Study

Construction of an oesophageal cancer-specific ceRNA network based on miRNA, lncRNA, and mRNA expression data

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Abstract

AIM

To explore the expression profiles of microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and mRNAs in oesophageal squamous cell carcinoma (ESCC) in order to construct an oesophageal cancer-specific competing endogenous RNA (ceRNA) network.

METHODS

In this work, the expression data of miRNAs, lncRNAs, and mRNAs in ESCC were obtained. An oesophageal cancer-specific ceRNA network was then constructed and investigated.

RESULTS

CeRNAs have the ability to reduce the targeting activity

of miRNAs, leading to the de-repression of specific mRNAs with common miRNA response elements. CeRNA interactions have a critical effect in gene regulation and cancer development.

CONCLUSION

This study suggests a novel perspective on potential oesophageal cancer mechanisms as well as novel pathways for modulating ceRNA networks for treating cancers.

Key words: Competing endogenous RNA; MicroRNA; Long non-coding RNA; mRNA; Oesophageal squamous cell carcinoma

Core tip: Competing endogenous RNAs (ceRNAs) may play a critical role in tumourigenesis, and perturbations to ceRNA networks would result in the progression of oesophageal squamous cell carcinoma (ESCC). However, the role of ceRNAs in ESCC has not been comprehensively explored. This study was designed to investigate the expression profiles of microRNAs, long non-coding RNAs, and mRNAs in ESCC to elucidate an oesophageal cancer-specific ceRNA network. Our report reveals potential molecular mechanisms of oesophageal cancer progression and suggests a novel approach to cancer therapeutics in the regulation of ceRNA networks.

Xue WH, Fan ZR, Li LF, Lu JL, Ma BJ, Kan QC, Zhao J. Construction of an oesophageal cancer-specific ceRNA network based on miRNA, lncRNA, and mRNA expression data. *World J Gastroenterol* 2018; 24(1): 23-34 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i1/23.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i1.23

INTRODUCTION

Oesophageal squamous cell carcinoma (ESCC) is the sixth leading death reason of cancer[1]. According to the official statistics in America, more than 18000 cases were newly diagnosed with 15000 deaths from oesophageal cancer in 2014, representing 5% of all cancer deaths[2]. Recently, the incidence and mortality rate of ESCC have decreased in North America and Europe[3]. However, ESCC has a significant ethnic and geographic distribution and it has been highly prevalent in China and other Asia countries. The presence of familial aggregation suggests that the risk factors for ESCC include environmental and genetic factors[4]. When ESCC is diagnosed, most patients have already progressed to be advanced or metastatic. Thus, as there is no longer an opportunity for radical surgery, radiation and chemotherapy become the major palliative treatments[5].

Tumourigenesis and cancer development have been closely associated with the aberrant expression of protein coding mRNAs and non-coding RNAs[6]. Approximately 98% of the human genome are non-coding RNAs, suggesting their promising effects on physiological and pathological processes[7]. MicroRNAs (miRNAs) suppress the translation and induces the degradation of mRNA, thus modulating gene expression and function[8]. MiRNAs have been proved to have critical effects in tumourigenesis, and the role of miRNAs has been relatively well understood[9]. Long non-coding RNAs (lncRNAs) are newly found non-coding RNAs which were proved to participate in many diseases[10]. However, the functional role of the large number of lncRNAs in ESCC remains unclear.

Many studies have confirmed that competing endogenous RNAs (ceRNAs) are able to act as sponges for miRNAs. The activity of miRNAs could be modulated with the variation of ceRNA abundance from individual genes[11]. Interactions between ceRNAs through sharing miRNAs indicate a new pathway of gene regulation, which has key effects in the cancer progression[12-14]. CeRNAs act as molecular sponges of miRNAs through binding with miRNAs (also known as miRNA response elements, MRE), thus inhibiting miRNA targeted genes[15]. The discovery of ceRNAs requires reassessing our understanding of gene regulatory networks and raising the probability of proposing a new molecular mechanism. Both of them may be the potential targets for gene treatment[16-18].

Lately, complex and multidimensional molecular maps of large cancer crowd were uncovered by research alliance such as The Cancer Genome Atlas (TCGA). With these information, a synthetic analysis could be performed on the association between molecular alterations and certain cancer type[19-21]. Many ceRNAs were revealed in various cancer types. Until now, few studies have been performed on clarifying the association among lncRNAs, miRNAs, and mRNAs in ESCC. Therefore, in this study, a ceRNA network in ESCC was constructed, which may help to elucidate the specific biological mechanisms of ESCC progression.

MATERIALS AND METHODS

Data sets and pre-processing

The expression data of miRNAs and mRNAs in 101 oesophageal cancer patients were collected from the National Center for Biotechnology Information Gene Expression Omnibus (NCBI) with login numbers of GSE45670[22] (38 patients, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE45670), GSE26886[23] (28 patients, http://www.ncbi.nlm.nih.Gov/geo/query/acc.cgi?acc=GSE26886), GSE17351 (10 samples, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE17351), GSE55856[24] (216 patients, http://www.ncbi.nlm.nih.gov/geo/query/

acc.cgi?acc=GSE55856), and GSE66274[25] (60 patients, http://www.ncbi.nlm.nih.Gov/geo/query/acc.cgi?acc=GSE66274). Various miRNA targets and oesophageal cancer data sets were also applied for assessing the reliability of this approach, aimed to construct a ceRNA network. Under these circumstances, we also implanted the expression profiles of 170 matched miRNAs and mRNAs in oesophageal cancer patients from TCGA[26]. Annotation information of lncRNAs was obtained with Affymetrix Human Genome U133 Plus 2.0 arrays. The network of protein-protein interactions was constructed using STRING database system.

Functional analysis

DAVID (Databases for Annotation, Visualization and Integrated Discovery) was included to determine the pathways of KEGG (Kyoto Encyclopedia of Genes and Genomes) and Gene Ontology (GO) Term biological processes were enriched with central genes recommunities in the ceRNA network. *P* values < 0.05 indicated enriched gene sets[27].

Network visualisation and community detection

The miRNA-lncRNA-mRNA interaction network was visualised with Cytoscape Software, and topology analysis was performed with network analyser plugin. MCODE plugin was also applied (with its default parameters) to figure out the communities (dense clusters) in the network[28].

Bioinformatics analysis on the associated expressions of lncRNAs, miRNA, and mRNAs

The single-stranded miRNAs would bind the mRNA transcripts, thus the post-transcriptional regulation of mRNA has been set up according to the relationships among miRNAs, lncRNAs, and mRNAs[29,30]. First, the miRNAs, lncRNAs, and mRNAs which were differentially expressed between ESCC specimens and corresponding normal tissues were chosen. The differential expression of miRNAs, lncRNAs, and mRNAs was identified with standard selection criteria, which were set at $P < 0.05$ and fold change > 2. In addition, the co-expression network of miRNAs, lncRNAs, and mRNAs was constructed according to the connections among the differentially expressed miRNAs, lncRNAs, and mRNAs.

Statistical analysis

Data are expressed as the mean ± SD. Student's *t*-test and analysis of variance were applied in the statistical analysis for comparing results of two groups and multiple groups, respectively[31]. The fold change and Student's *t*-test were applied to analyse the significance of microarray analysis. $P < 0.05$ indicated a statistically significant difference. *P* value was corrected with false discovery rate. The differentially expressed lncRNAs, miRNAs, and mRNAs are expressed as fold change values ($P < 0.05$).

RESULTS

Clustering analysis

We used unsupervised hierarchical clustering analysis in this study. Cases were organized by clustering analysis on the basis of immunostaining profiles, and cases were placed together with similar immune profiles as neighbouring rows in a clustergram. The dendrogram was applied to demonstrate the relationship among cases and immune markers. The branch length of dendrogram indicated the correlations in immunostaining results. The unsupervised hierarchical cluster analysis demonstrated the correlation of expression maps between biological replicates and group conditions (Figure 1A-C).

Cancer-specific lncRNAs, miRNAs, and mRNAs in ESCC

The inter-connected complexity of physiological, cellular, and molecular functions has increasingly grown, thus novel approaches are required to simultaneously demonstrate multiple datasets[32]. There are multiple intersecting regions (generally as circles) in Venn diagram, which enables the description of all logical relations among various data sets[33]. Here, we selected 21 miRNAs from GSE66274 and GSE55856; 228 mRNAs from GSE26886, GSE17351, and GSE45670; and 31 lncRNAs from GSE26886, GSE17351, and GSE45670 (Figure 2A-C).

mRNA GO analysis in ESCC

In the GO database, there are structured, controlled vocabularies and classifications covering several molecular and cellular biology domains. GO has been applied for the annotation of genes and sequences[34].

The 228 genes with differential expression were analysed with the GO database. The enrichment of these genes was analysed in specific pathways. Enrichment analysis is used to evaluate the significance of the function, which helps provide GO terms with a more definitive function demonstration[35]. As shown in Table 1, the most highly enriched GO path was 'extracellular matrix organization'. The genes in 'extracellular matrix organization' path were MMP3, MMP10, LAMA3, MMP9, MMP13, COL11A1, BMP7, MMP12, LAMC2, COL27A1, ITGB4, PDGFRA, ADAMTS2, IBSP, COL10A1, COL7A1, MMP11, MFAP2, MMP1, and COL1A1. The second most highly enriched GO path was 'collagen catabolic process' (Figure 3).

mRNA pathway analysis in ESCC

KEGG systematically interprets sequence data by computerizing biochemical pathways and other types of molecular interactions[36-38]. The results showed that the most highly enriched pathway was 'transcriptional misregulation in cancer' (Table 2). The genes in

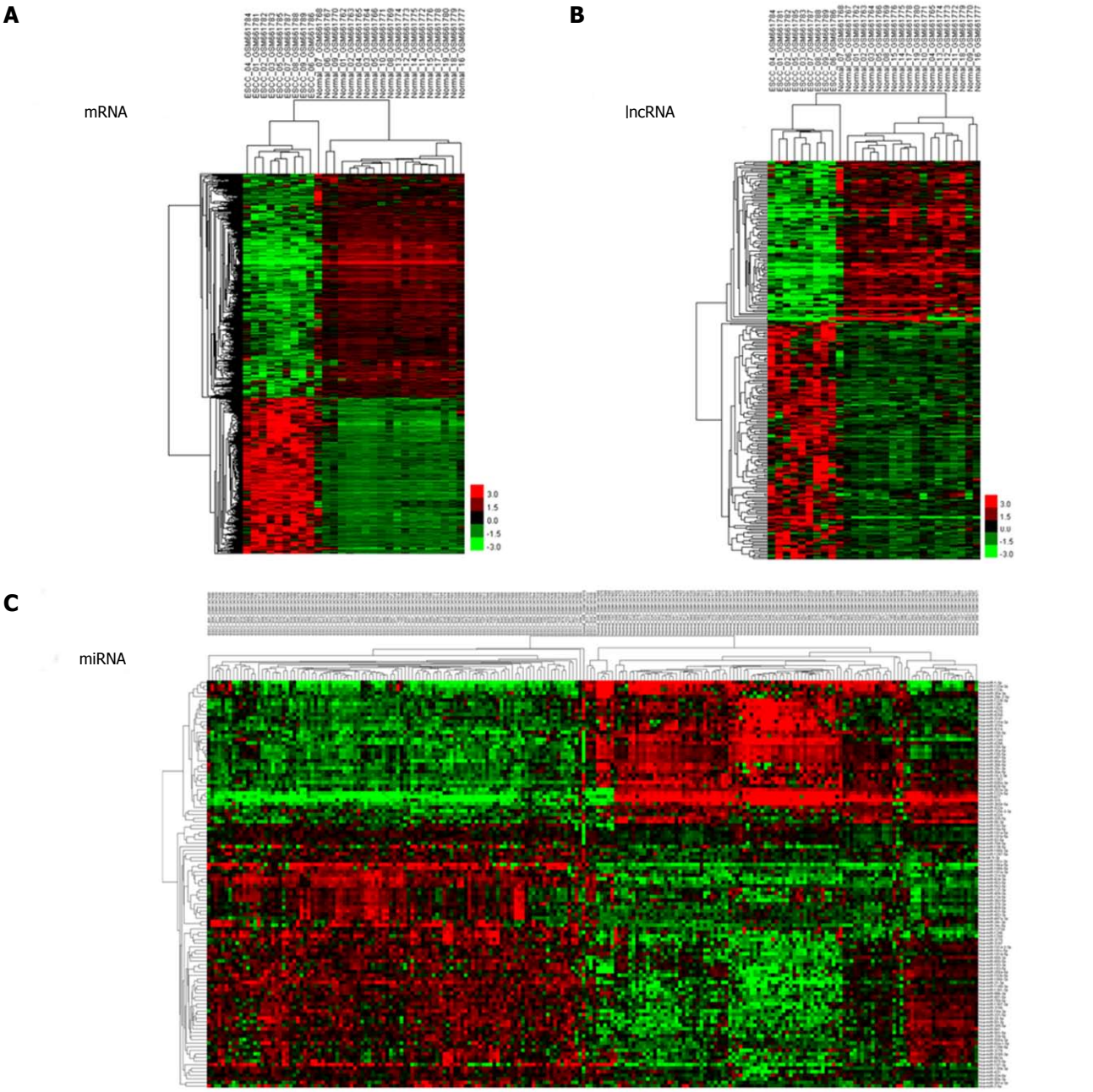

Figure 1 Cluster analysis of differentially expressed profiles. A: mRNAs; B: lncRNAs; and C: miRNAs in tumour tissues *vs* adjacent non-tumour tissues. The result of hierarchical cluster analysis shows distinguishable expression profiles between samples. The rows show differentially expressed miRNAs, lncRNAs, and mRNAs, while the columns show three paired samples. Red represents high expression and green represents low expression.

'transcriptional misregulation in cancer' pathway were TCF3, CXCL8, SIX1, IGFBP3, MLF1, PLAU, MEIS1, HOXA10, MMP9, SIX4, HPGD, and MMP3. The second most highly enriched pathway was 'ECM-receptor interaction' (Figure 4). The genes in 'ECM-receptor interaction' pathway were ITGB4, COL1A1, COL11A1, ITGA3, LAMC2, SPP1, LAMB3, and IBSP.

Protein regulation network analysis

Protein-protein interactions have been not only direct binding, but also indirect actions[39]. Genomic associations between protein-coding genes are provided for interring functional links between proteins. Genes that have the same function are often located in close to each other and tend to participate in gene-fusion events[40-42]. The database STRING has been used to analyse these associations[43]. We input the shared differential mRNAs from GSE45670, GSE26886, and GSE17351 into the STRING database. Several nodes with high degrees were COL27A1, COL7A1, COL1A1, ITGB4, ITGA3, SERPINE1, MMP1, MMP9, and MMP10 (Figure 5).

ceRNA network analysis

CeRNAs share common MRE and hence regulate RNA transcripts by competitively binding with general

Table 1 mRNA GO analysis in oesophageal squamous cell carcinoma

Goid	GO name	GO diffgene count	GO gene count	Enrichment	*P* value	FDR
GO:0030198	Extracellular matrix organization	20	210	25.11013216	1.54276E-21	1.68623E-18
GO:0030574	Collagen catabolic process	12	72	43.94273128	3.32314E-16	1.8161E-13
GO:0022617	Extracellular matrix disassembly	11	79	36.71164892	5.27621E-14	1.9223E-11
GO:0045944	Positive regulation of transcription	18	708	6.7031285	1.3435E-09	3.6711E-07
GO:0007155	Cell adhesion	14	454	8.130373188	1.16162E-08	2.30356E-06
GO:0044281	Small molecule metabolic process	23	1363	4.449080643	1.26454E-08	2.30356E-06
GO:0008285	Negative regulation of cell prolifer	12	358	8.837644279	6.42989E-08	1.00398E-05
GO:0001501	Skeletal system development	8	127	16.60827639	1.37682E-07	1.88108E-05
GO:0048699	Generation of neurons	4	11	95.87505006	2.60527E-07	3.16396E-05
GO:0008284	Positive regulation of cell prolifera	12	411	7.69799672	2.89703E-07	3.16645E-05
GO:0007165	Signal transduction	18	1030	4.607587357	4.19668E-07	4.16997E-05
GO:0055085	Transmembrane transport transmembrane transport	13	538	6.370879256	7.27191E-07	6.6235E-05
GO:0007566	Embryo implantation	5	37	35.62924158	1.18601E-06	9.97159E-05
GO:0048704	Embryonic skeletal system morph	5	40	32.95704846	1.77383E-06	0.000131776
GO:0006508	Proteolysis	12	488	6.483353795	1.80845E-06	0.000131776
GO:0008544	Epidermis development	6	76	20.81497797	1.95203E-06	0.000133348
GO:0048015	Phosphatidylinositol-mediated sign	7	129	14.30693576	2.77867E-06	0.000178652
GO:0043065	Positive regulation of apoptotic pr	8	197	10.70685838	3.9876E-06	0.000242136
GO:0019369	Arachidonic acid metabolic process	5	50	26.36563877	5.53803E-06	0.000318583
GO:0006979	Response to oxidative stress	6	101	15.6627557	1.04573E-05	0.000571494

Table 2 mRNA pathway analysis in oesophageal squamous cell carcinoma

Path_id	Path_name	Path_diffgene_count	Path_gene_count	Enrichment	*P* value	FDR
05202	Transcriptional misregulation in cancer	12	179	17.67528856	2.34961E-11	3.94734E-09
04512	ECM-receptor interaction	9	87	27.27479872	2.19839E-10	1.84664E-08
04510	Focal adhesion	10	207	12.73702356	3.3619E-08	1.79605E-06
04151	PI3K-Akt signalling pathway	12	345	9.170656962	4.27632E-08	1.79605E-06
05146	Amoebiasis	7	108	17.08883994	8.29201E-07	2.78612E-05
01100	Metabolic pathways	19	1234	4.059539194	1.27125E-06	3.5595E-05
05200	Pathways in cancer	11	397	7.305340716	1.71545E-06	4.11707E-05
04810	Regulation of actin cytoskeleton	8	214	9.856313558	7.4028E-06	0.000155459
00590	Arachidonic acid metabolism	5	62	21.26261191	1.62994E-05	0.000304255
04115	p53 signalling pathway	5	68	19.38649909	2.57756E-05	0.00043303
04060	Cytokine-cytokine receptor interaction	8	265	7.959438118	3.5607E-05	0.000543816
04974	Protein digestion and absorption	5	90	14.64757709	0.000101556	0.001421784
04666	Fc gamma R-mediated phagocytosis	5	92	14.3291515	0.000112939	0.001459525
05205	Proteoglycans in cancer	6	203	7.792799635	0.000540094	0.006142299
04610	Complement and coagulationcasc	4	69	15.28442827	0.000574387	0.006142299
04611	Platelet activation	5	130	10.14063029	0.000584981	0.006142299
05132	Salmonella infection	4	86	12.2630878	0.001341668	0.01252223
05222	Small cell lung cancer	4	86	12.2630878	0.001341668	0.01252223
05323	Rheumatoid arthritis	4	89	11.84972529	0.001529001	0.01351959
00564	Glycerophospholipid metabolism	4	95	11.10132159	0.001958567	0.016451962

miRNA molecules[44]. CeRNAs could be relieved from miRNA-mediated repression and their expression levels could be positively modulated[45]. The discovery of ceRNAs provides many implications for cancer, which have already been extensively discussed[46].

Based on the expression profiles of specific miRNAs, lncRNAs, and mRNAs in patients with oesophageal cancer, a ceRNA network was constructed using a computational method proposed for this study (Figure 6) and it was drawn with Cytoscape 3.0[47]. The ceRNA network has integrated the miRNA-lncRNA-mRNA interactions by negative regulation.

There are 74 nodes in the oesophageal cancer-specific ceRNA network. The degrees of the hsa-miR-93-5p, hsa-miR-34c-5p, and hsa-miR-18a-3p nodes were 14, 12, and 11, respectively. The density of our ceRNA network was confirmed with the high degree of nodes, suggesting common competitions among RNAs for oesophageal cancer. The modes degree was also observed to follow power law distribution. For miRNAs, the expression of hsa-miR-196b-5p, has-miR-34c-5p, and has-miR-18a-3p were up-regulated. However, the expression levels were down-regulated for has-miR-30a-3p, has-miR-150-5p, and has-miR-133a-3p. All these analysis results suggest the scale-free ceRNA network in oesophageal cancer and the biological significance may be reflected by the topological structures including the hubs, nodes, and

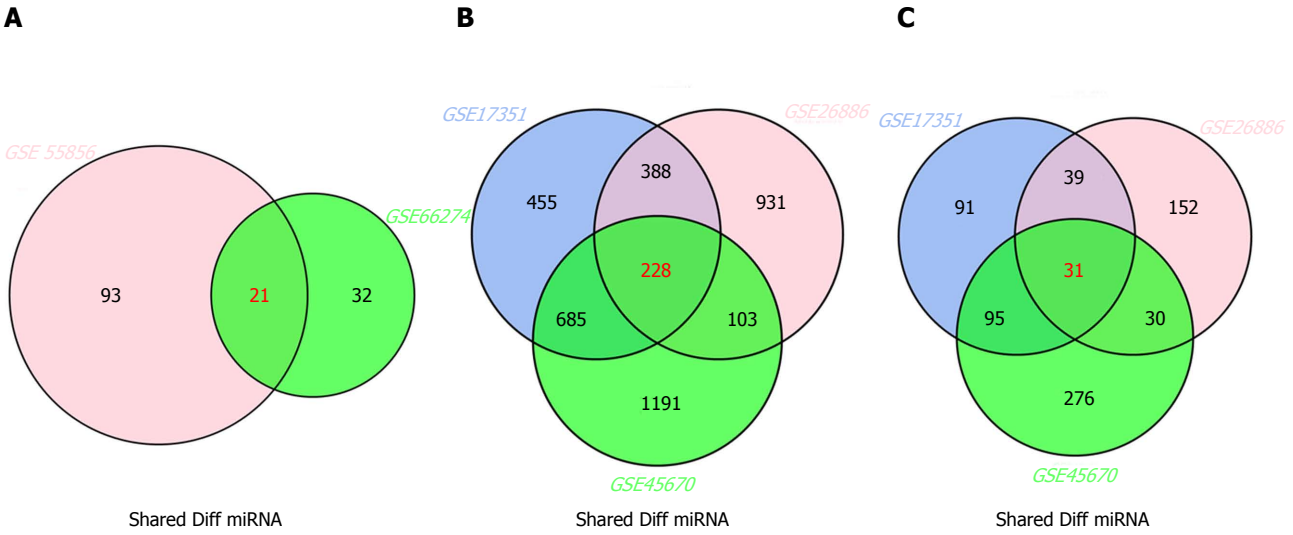

Figure 2 Venn diagram analysis of differentially expressed genes in comparison groups. A: miRNAs; B: mRNAs; C: lncRNAs.

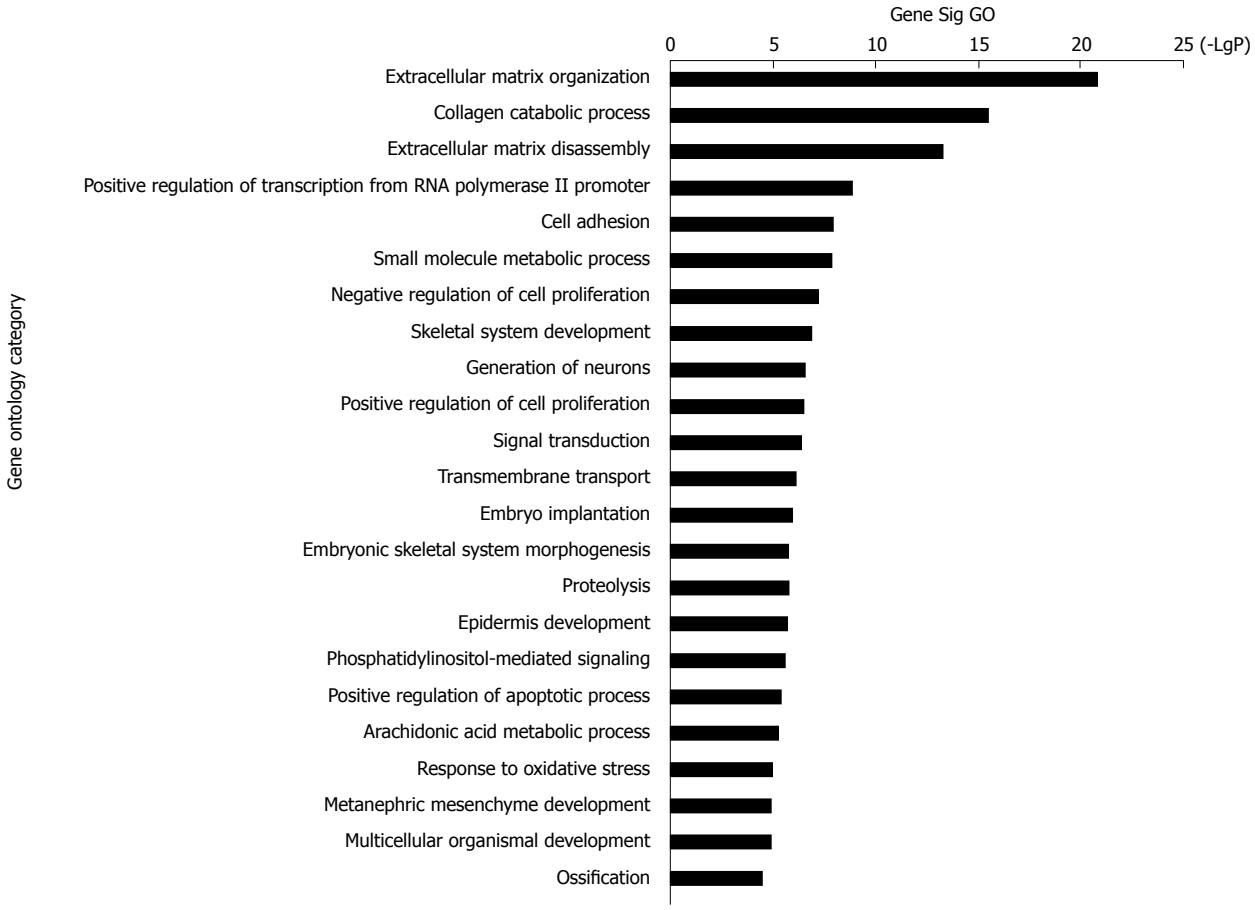

Figure 3 Top 23 GO enrichment terms for differentially expressed intersection mRNAs. GO analysis of the common differentially expressed mRNAs was performed.

communities.

mRNA survival curves

To further identify the key mRNAs that were associated with prognostic characteristics in 170 ESCC patients, the overall survival was profiled with the univariate Cox proportional hazards regression model ($P < 0.05$). Among the six significant mRNAs, the overall survival was negatively related to five mRNA transcripts (STC2, SLC6A1, MMP12, EPCAM, and EPB411L4B) ($P < 0.05$) while positively associated with the remaining mRNA transcript (LAMC2) ($P < 0.05$) (Figure 7A-F).

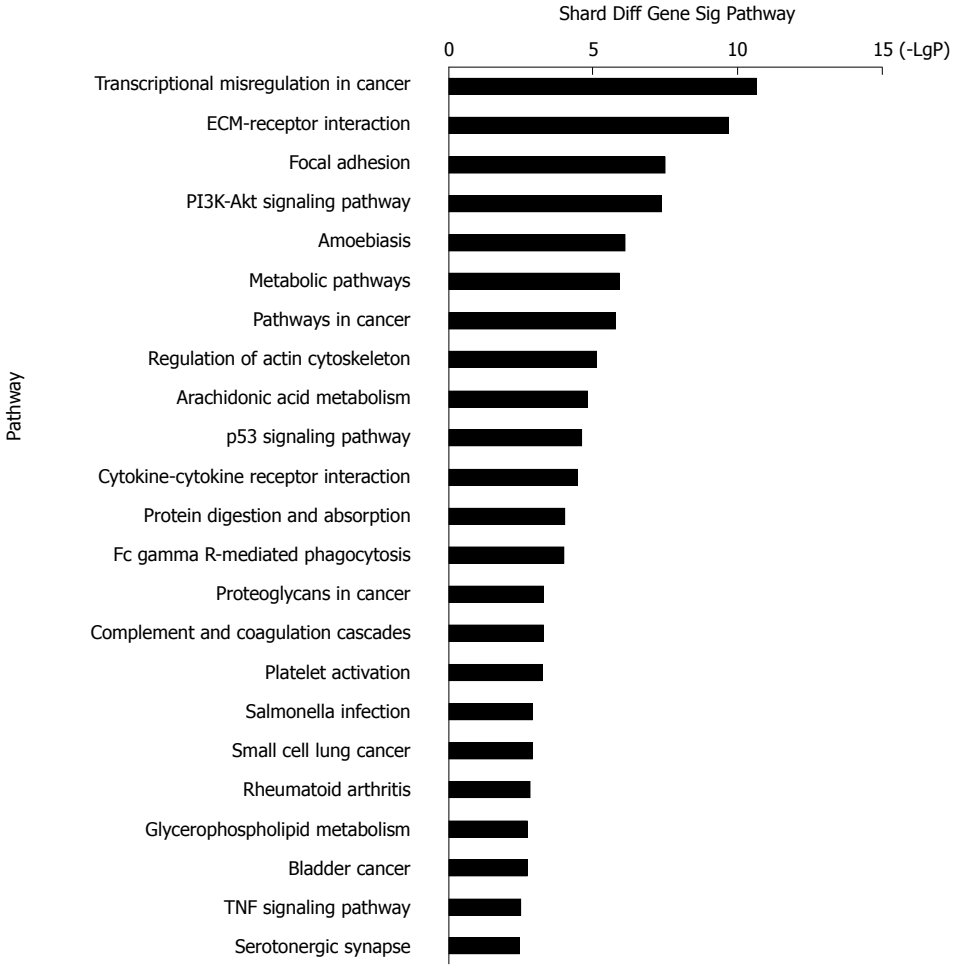

Figure 4 Top 23 pathway enrichment terms for differentially expressed intersection mRNAs. KEGG pathway analysis of the common differentially expressed mRNAs was performed.

DISCUSSION

It is necessary to explore ceRNA cross-talk across multiple cancer types[48]. TCGA was formed to meet these needs and its vast data sets provide us with an unprecedented opportunity to systematically analyse the ceRNA network in cancer. These interesting findings led us to construct an oesophageal cancer-specific ceRNA network.

In this work, clustering analysis, mRNA GO analysis, mRNA pathway analysis, and protein regulation network analysis in ESCC were conducted to construct the ceRNA network. The results showed that the most highly enriched GO path was 'extracellular matrix organization'. The genes in 'extracellular matrix organization' path were MMP3, MMP10, LAMA3, MMP9, MMP13, COL11A1, BMP7, MMP12, LAMC2, COL27A1, ITGB4, PDGFRA, ADAMTS2, IBSP, COL10A1, COL7A1, MMP11, MFAP2, MMP1, and COL1A1. Advances in structural genomics will make it possible to reveal the complete genome sequence of hundreds of organisms. The ceRNA network analysis indicated that the degree of has-miR-93-5p as an up-regulated gene was 14. All these results are relevant to the further development of treatments for oesophageal cancer.

Based on Kaplan-Meier analysis, overall survival was negatively related to five mRNA transcripts (STC2, SLC6A1, MMP12, EPCAM, and EPB41L4B) ($P < 0.05$) and it was positively associated with the remaining mRNA transcript (LAMC2) ($P < 0.05$). These mRNAs could be candidate and specific biomarkers for the diagnosis, prognosis, and classification of ESCC.

In this research, a computational approach has been proposed for the construction of ceRNA network based on existing data of esophageal cancer. In this network, the junction nodes indicate paired gene pair in competing mRNA library. We observed that the ESCC-specific ceRNA network is scale-free, and the dense clusters in the network are associated with promising markers. The results of mRNA pathway analysis showed that the most highly enriched pathway was transcriptional misregulation in cancer. In addition, overall survival was negatively related to the genes STC2, SLC6A1, MMP12, EPCAM, and EPB41L4B, while it was positively associated with LAMC2. These confirmed results suggested that the

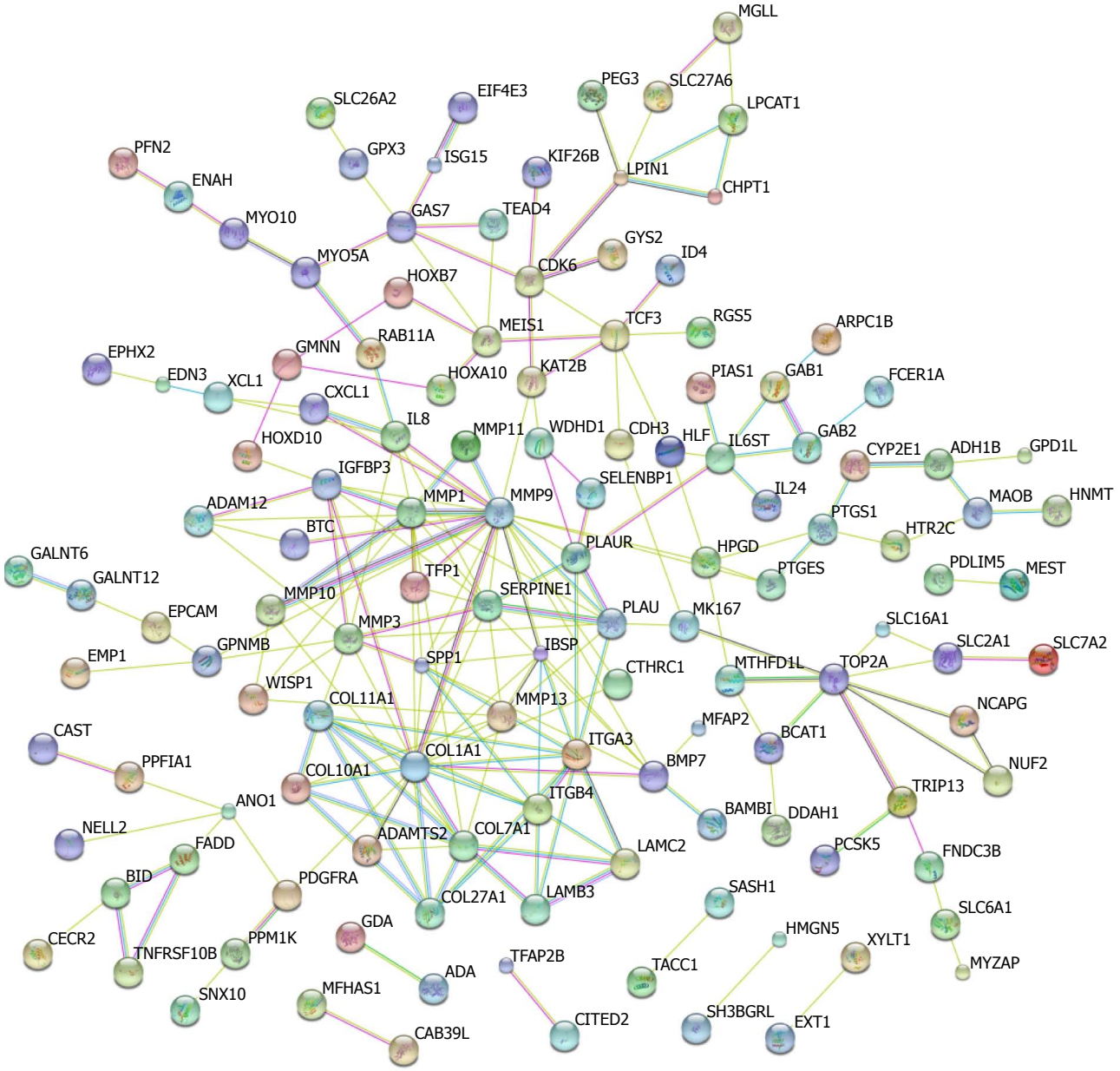

Figure 5 Protein regulation network analysis. The protein-protein interaction networks were constructed by Cytoscape Software. Proteins are represented with colour nodes, and interactions are represented with edges.

biological mechanism of ESCC could be discovered with the constructed ceRNA network. Importantly, a simple framework has been provided in our work for the construction of a ceRNA network, which can be used to a variety of biological issues, such as ESCC and its biological processes. In short, cancer-specific miRNAs, lncRNAs, and mRNAs in ESCC can be successfully identified in the present study by bioinformatics analysis from large scale samples. Moreover, understanding the ceRNA network in ESCC may reveal potential intended targets for cancer sub-populations or across cancers. This work suggests new approaches for studying the role and mechanism of ceRNAs in human cancers using publicly available genomic data.

ARTICLE HIGHLIGHTS

Research background

Oesophageal squamous cell carcinoma (ESCC) is one of the most prevalent forms of oesophageal cancer, and its development is closely related to the abnormal expression of not only protein-encoding mRNAs, but also non-coding RNAs. Competitive endogenous RNAs (ceRNAs) regulatory networks include mRNAs, miRNAs, lncRNAs, and circular RNAs, which participate in the cancer pathogenesis by regulating each other's expression. However, their function

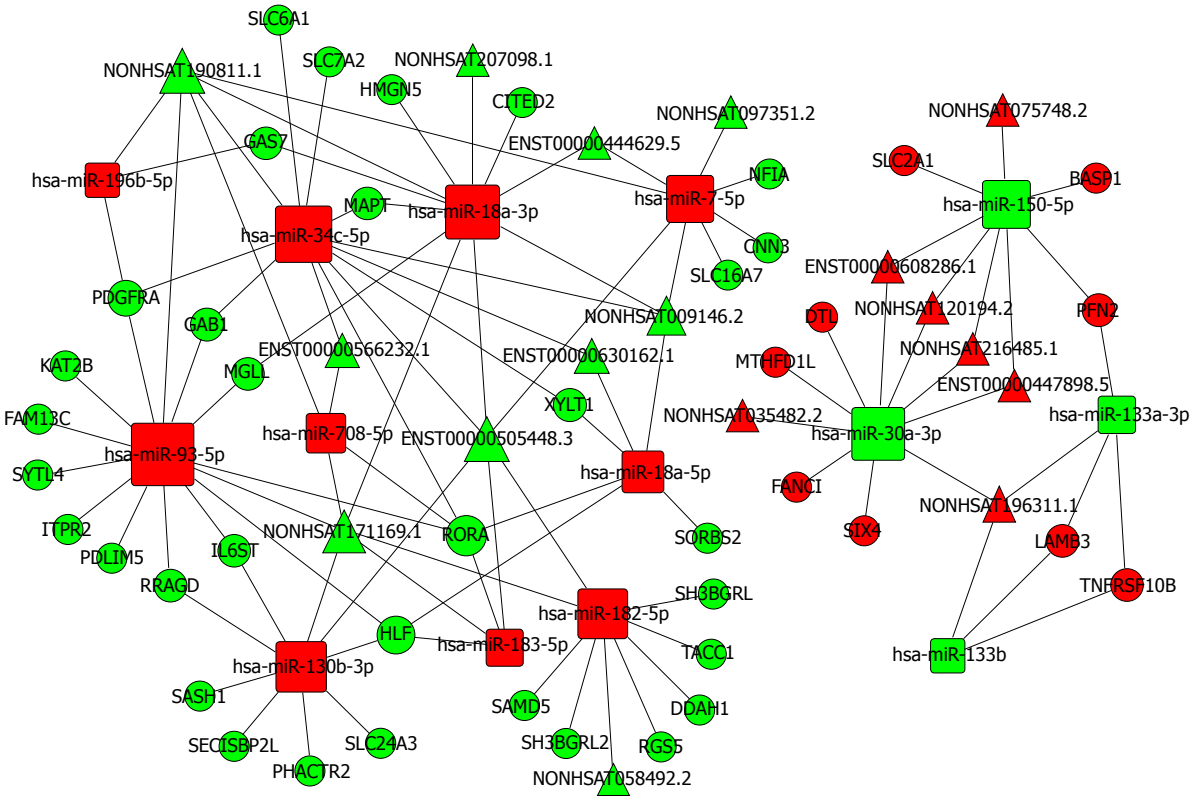

Figure 6 The lncRNA-miRNA-mRNA ceRNA network. The rectangles indicate miRNAs and circles represent mRNAs. The red indicates up-regulation and green indicates down-regulation.

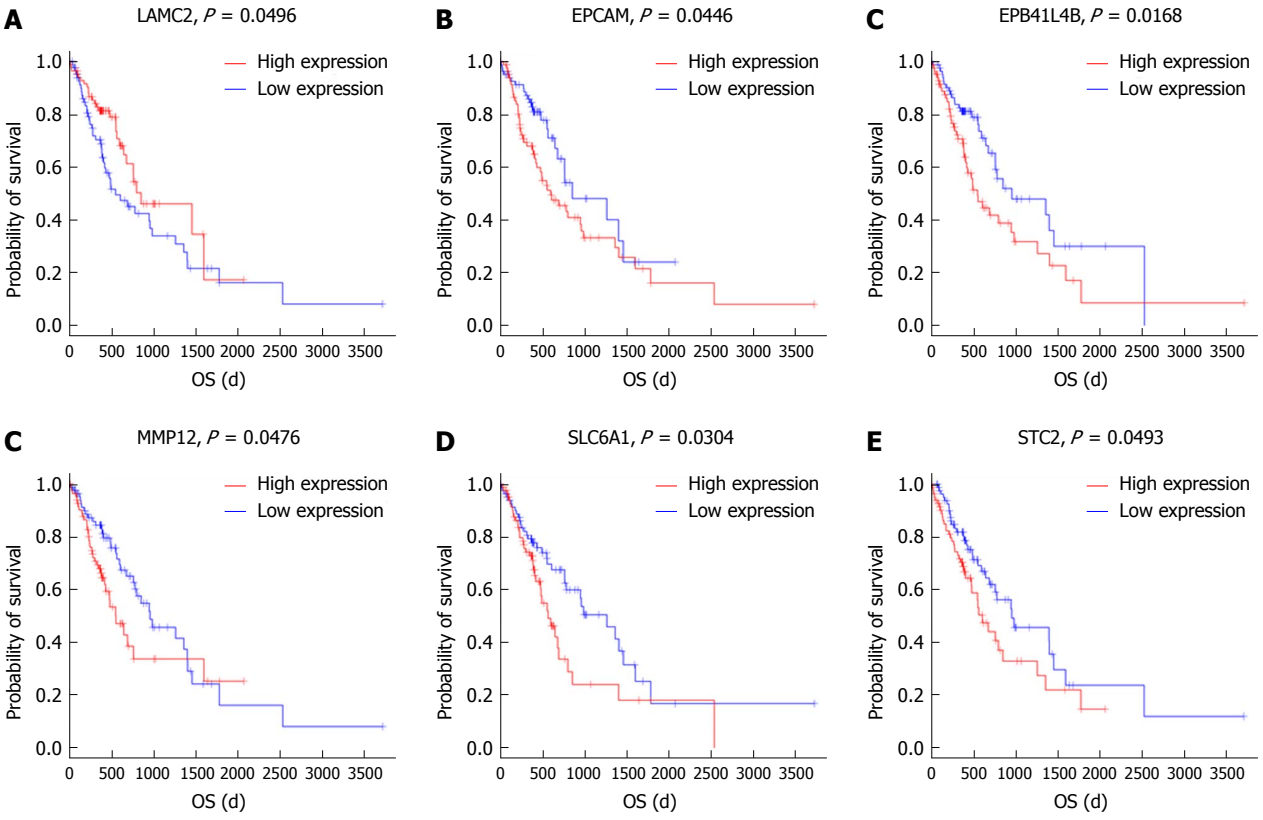

Figure 7 Kaplan-Meier survival curves for eight mRNAs associated with overall survival. Log-rank tests were performed to evaluate the survival differences between the two curves. Horizontal axis: Overall survival time, days; Vertical axis: Survival function.

has not been clarified in ESCC. Therefore, construction of a ceRNA network for ESCC may help to study the biological mechanisms of this malignancy.

Research motivation

It is necessary to explore the CeRNA cross-talk across multiple cancer types. These issues have been addressed by TCGA, which provides large data sets enabling us with an unprecedented opportunity to synthetically explore the ceRNA network for various cancers. These findings led us to construct an oesophageal cancer-specific ceRNA network. The present study found that there were mRNAs, miRNAs, and lncRNAs in the ceRNA regulatory network, which might play a critical role in ESCC, and the abnormality in ceRNA regulatory networks would lead to the initiation and progression of ESCC.

Research objectives

Clustering analysis, mRNA GO analysis, mRNA pathway analysis, and protein regulation network analysis in oesophageal squamous cell carcinoma were conducted to construct a ceRNA network. These confirmed results suggested that the biological mechanisms in the development of ESCC may be indeed associated with the ceRNA network. Importantly, a simple framework was proposed in this study for constructing ceRNA networks in various biological processes including the study on ESCC.

Research methods

The expression data of miRNAs and mRNAs in 101 patients with esophageal cancer were obtained from the National Center for Biotechnology Information Gene Expression (NCBI). The expression profiles of 170 matched miRNAs and mRNAs in esophageal cancer patients were also obtained from TCGA (The Cancer Genome Atlas). The KEGG pathway and GO Term biological processes were identified with DAVID. The results were drawn with Cytoscape software, and were topologically analysed by Cytoscape's network analyzer plugin. In addition, communities (dense clusters) in the network was found with Cycloscape, using the MCODE plug-in (the default). Based on the relationship between miRNAs, lncRNAs, and mRNAs, strands of stranded miRNAs have been established following transcriptional regulation of single nucleotide sequence-associated mRNA transcripts.

Research results

The results showed that the most highly enriched Gene Ontology path was 'extracellular matrix organization'. The genes in 'extracellular matrix organization' path were MMP3, MMP10, LAMA3, MMP9, MMP13, COL11A1, BMP7, MMP12, LAMC2, COL27A1, ITGB4, PDGFRA, ADAMTS2, IBSP, COL10A1, COL7A1, MMP11, MFAP2, MMP1, and COL1A1. The advances in structural genomics may reveal the complete genomic sequence of thousands of organisms. The ceRNA network analysis indicated that the degree of has-miR-93-5p as an up-regulated gene was 14. All these results are meaningful for further development of treatments for oesophageal cancer. The overall survival was negatively associated with five mRNAs (STC2, SLC6A1, MMP12, EPCAM, and EPB41L4B), and it was positively related to the remaining mRNA (LAMC2). These mRNAs can be applied as promising specific biomarkers for ESCC. The significantly dysregulated mRNAs and miRNAs need to be validated in the future.

Research conclusions

A ceRNA network was identified in ESCC. The overall survival was negatively related to five mRNAs (STC2, SLC6A1, MMP12, EPCAM, and EPB41L4B). The ceRNA network has a significant effect in gene regulation and cancer development in ESCC. This study provides potential mechanisms for the development of oesophageal cancer and suggests new methods to modulate ceRNA networks for cancer treatment. CeRNA networks are implicated in the development of ESCC. A relationship between lncRNAs, miRNAs, and mRNAs in oesophageal squamous cell carcinoma was constructed by bioinformatics analysis. Cytoscape software showed the miRNA-lncRNA-mRNA interaction network and the Cytoscape network analyzer plug-in was used for topology analysis. In addition, the communities (dense clusters) in the network were found with the MCODE plug-in (with the default parameters). The bioinformatics analysis was performed on the co-expression of lncRNAs, miRNA, and mRNAs. The results showed that the most highly enriched GO path was 'extracellular matrix organization', which was associated with ESCC. By examining the ceRNA network, the node degrees were observed to follow a power law distribution. The expression of hsa-miR-196b-5p, has-miR-34c-5p, and has-miR-18a-3p was up-regulated. However, the levels of has-miR-30a-3p, has-miR-150-5p, and has-miR-133a-3p were down-regulated. The ceRNA network is associated with cancer progression. The understanding of ceRNA networks in ESCC may help uncover unexpected potential therapeutic targets that would be available in cancer sub-populations or across cancers.

Research perspectives

Understanding the ceRNA network is of significance in identifying potential therapeutic targets for ESCC. Our study focuses on the function and mechanism of ceRNAs in ESCC using publicly available genomic data.

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ORIGINAL ARTICLE

Basic Study

Emodin and baicalein inhibit sodium taurocholate-induced vacuole formation in pancreatic acinar cells

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Abstract

AIM

To investigate the effects of combined use of emodin and baicalein (CEB) at the cellular and organism levels

in severe acute pancreatitis (SAP) and explore the underlying mechanism.

METHODS

SAP was induced by retrograde infusion of 5% sodium taurocholate into the pancreatic duct in 48 male SD rats. Pancreatic histopathology score, serum amylase activity, and levels of tumour necrosis factor alpha (TNF-α), interleukin 6 (IL-6), and IL-10 were determined to assess the effects of CEB at 12 h after the surgery. The rat pancreatic acinar cells were isolated from healthy male SD rats using collagenase. The cell viability, cell ultrastructure, intracellular free Ca^{2+} concentration, and inositol (1,4,5)-trisphosphate receptor (IP_3R) expression were investigated to assess the mechanism of CEB.

RESULTS

Pancreatic histopathology score (2.07 ± 1.20 *vs* 6.84 ± 1.13, $P < 0.05$) and serum amylase activity (2866.2 ± 617.7 *vs* 5241.3 ± 1410.0, $P < 0.05$) were significantly decreased in the CEB (three doses) treatment group compared with the SAP group (2.07 ± 1.20 *vs* 6.84 ± 1.13, $P < 0.05$). CEB dose-dependently reduced the levels of the pro-inflammatory cytokines IL-6 (466.82 ± 48.55 *vs* 603.50 ± 75.53, $P < 0.05$) and TNF-α (108.04 ± 16.10 *vs* 215.56 ± 74.67, $P < 0.05$) and increased the level of the anti-inflammatory cytokine IL-10 (200.96 ± 50.76 *vs* 54.18 ± 6.07, $P < 0.05$) compared with those in the SAP group. CEB increased cell viability, inhibited cytosolic Ca^{2+} concentration, and significantly ameliorated intracellular vacuoles and IP_3 mRNA expression compared with those in the SAP group ($P < 0.05$). There was a trend towards decreased IP_3R protein in the CEB treatment group; however, it did not reach statistical significance ($P > 0.05$).

CONCLUSION

These results at the cellular and organism levels reflect a preliminary mechanism of CEB in SAP and indicate that CEB is a suitable approach for SAP treatment.

Key words: Inositol (1,4,5)-trisphosphate receptor; Severe acute pancreatitis; Calcium overload; Emodin; Baicalein; Pancreatic acinar cell

Core tip: Combined use of emodin and baicalein (CEB) was found to act on key aspects of severe acute pancreatitis (SAP) pathogenesis; it regulated inflammatory factors and inhibited calcium overload, which underlie this synergy. These results at the cellular and organism levels reflect a preliminary mechanism of CEB in SAP and indicate that the development of CEB is promising for the treatment of patients with SAP.

Li J, Zhou R, Bie BB, Huang N, Guo Y, Chen HY, Shi MJ, Yang J, Zhang J, Li ZF. Emodin and baicalein inhibit sodium taurocholate-induced vacuole formation in pancreatic acinar cells. *World J Gastroenterol* 2018; 24(1): 35-45 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i1/35.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i1.35

INTRODUCTION

Severe acute pancreatitis (SAP) is a debilitating disease that is characterized by an acute onset, serious complications, and significant morbidity and mortality[1]. Currently, the somatostatin analogue octreotide is the most frequently used drug in SAP therapy[2,3]. However, not all patients are treated with octreotide due to its short half-life *in vivo* and high cost. Identifying other safe, effective, and inexpensive drugs for the treatment of SAP remains necessary.

Traditional Chinese medicines (TCMs) have shown efficacy in the management of many inflammatory diseases[4]. The most famous formula for the treatment of SAP is Qingyi decoction, which is composed of rheum, Chinese thorowax root, white peony root, baikal skullcap root, coptis chinensis, and other herbs and has been advocated for more than one hundred years in China[5,6]. Qingyi decoction has exhibited good therapeutic effects in individuals with SAP in many clinical and pre-clinical studies[7-9]. However, use of this decoction is limited because patients are not allowed access to food and water in the acute stage. Furthermore, the formulation of this TCM is difficult to standardize due to the large number of herbs in one decoction. Guided by the theories of TCM and modern medicine and the compatibility of herbs, we refined Qingyi decoction and combined emodin and baicalein into "Compound Emodin and Baicalein" (CEB, patent No. ZL200310105814.3).

Emodin and baicalein are the active ingredients in rheum and baikal skullcap root and have an identified chemical constitution[10,11]. Emodin has been reported to block the development of SAP[12]. In our previous study, we were the first to show that baicalein protected against pancreatic injury in rats with SAP and exhibited inhibitory effects on pro-inflammatory cytokine production[11]. Furthermore, CEB had a beneficial effect on SAP in rats, which is similar to the effect of octreotide[13-15]. However, the underlying mechanisms remain unknown. We aimed to investigate the above problems, which are crucial for the study and development of CEB. Here, we report that CEB exhibited a good effect on SAP rats, decreased the serum levels of pro-inflammatory cytokines, and increased the levels of anti-inflammatory factors *in vivo*. CEB attenuated inositol (1,4,5)-trisphosphate receptor (IP_3R) mRNA expression and inhibited calcium overload in pancreatic acinar cells. Therefore, we suggest that CEB might be a novel drug for SAP treatment in the future.

Table 1 Criteria for microscopic assessment

Histological pattern	Assessment	Score
Edema	Mild	1
	Moderate	2
	Severe	3
Inflammatory infiltration	Mild	1
	Moderate	2
	Severe	3
Fat necrosis	< 2/section	3
	3-5/section	5
	> 5/section	7
Parenchymal necrosis	Focal (< 5%)	3
	And/or sublobular (< 20%)	5
	And/or lobular (> 20%)	7
Hemorrhage	Mild	3
	Moderate	5
	Severe	7

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley (SD) rats (weighing 220 ± 10 g) were obtained from the Experimental Animal Center of Xi'an Jiaotong University Health Science Center and were housed at 23 ± 2 ℃ under a 12-h light/dark cycle. The animals were fasted overnight before the start of the experiments with free access to water. All animal procedures were approved by the Institutional Animal Care and Use Committee of Xi'an Jiaotong University Health Science Center and performed according to the National Guide for the Care and Use of Laboratory Animals.

Induction of SAP

SAP was induced *via* a retrograde infusion of 5% sodium taurocholate into the pancreatic duct as previously described[16]. The animals were anaesthetized with sodium pentobarbital (50 mg/kg, Sigma-Aldrich), and the abdomen was opened *via* a midline incision. First, we identified the common bile duct and duodenum. The pancreatic bile duct was occluded with two microvascular clamps to prevent reflux. In total, 5% sodium taurocholate was injected into the pancreatic duct at a dose of 0.1 mL/100 g body weight (at an injection velocity of 0.1 mL/min). Then, the injection site was pressed for 3 min and the surgery was completed by abdominal stratified closing. Special attention was focused on the atraumatic surgical technique. The humane killing was performed 12 h after the operation to determine the severity of SAP.

Therapeutic effects of CEB

In total, SAP was induced in 48 SD rats *via* a retrograde injection of 5% sodium taurocholate into the common biliopancreatic duct. All SAP rats were randomly divided into four groups, including an SAP model group and three CEB groups (the low-dose group received a 1.6 mg/kg dose of emodin combined with a 3.5 mg/kg dose of baicalein; the middle-dose group received a 3.2 mg/kg dose of emodin combined with a 7 mg/kg dose of baicalein; and the high-dose group received a 6.4 mg/kg dose of emodin combined with a 14 mg/kg dose of baicalein). An additional 24 normal SD rats were separated into two groups: a normal group and a sham operation (SO) group. The normal group did not undergo the procedure. In the SO group, the incisions were closed immediately after turning over the pancreas. CEB was injected *via* the tail vein to animals of the three CEB treatment groups immediately after surgical operation. SO and SAP rats were given the same amount of normal saline. Pancreatic histopathology score, serum amylase activity, and levels of tumour necrosis factor-alpha (TNF-α), interleukin (IL)-6, and IL-10 were determined 12 h after the surgery.

Histology

The pancreatic tissue was fixed in 4% paraformaldehyde for 24 h, dehydrated, and embedded in paraffin using a routine protocol as previously described[11]. The embedded tissue was sliced at a 4-μm thickness and stained using haematoxylin and eosin (H&E). The histology scores of the sections were independently evaluated by two experienced investigators who were blinded to the group assignment. Ten random fields per slide were examined in the histopathological analysis of each pancreas. The grading criteria were as follows: oedema, acinar necrosis, haemorrhage, and inflammatory cell infiltration (Table 1). The final score of each section was the summation of each pathological parameter[17]. Finally, the mean ± SD was calculated (2 slides/rat, ⩾ 6 rats/group).

Enzyme activity and cytokine level measurement

Serum amylase activity was measured using an automatic biochemistry analyser (Roche, Basel, Switzerland). Serum IL-6, TNF-α, and IL-10 levels were determined using enzyme-linked immunosorbent assay kits (Neobioscience Biotech, Shenzhen, China and eBioscience, San Diego, USA) according to the manufacturers' protocols. The absorbance was read at 450 nm (620 nm as the reference wavelength) using a microplate reader (BioTek Inc., Winooski, VT, United States).

Isolation of pancreatic acinar cells from healthy rats

Pancreatic acinar cells were isolated as described by Williams *et al*[18] with slight modifications. Briefly, the pancreas was incubated with 5 mL of digestion solution, consisting of collagenase (NB8 Broad Range, 0.1 g/L, Serva) and a trypsin inhibitor (0.1 g/L, Sigma). The digestion solution was injected (at multiple loci) into the pancreatic parenchyma until it was well distended. After 10 min, the pancreas was cut into 2-4-mm pieces, followed by shaking for 15 min

in a 37 ℃ water bath (120 cycles per minute). Next, 5 mL of fresh digestion solution was added to the pancreas. The digestion period was repeated 3 times, and the liquids were then collected and combined. The tissue was then gently pipetted through the pipette tip. After the filtration and centrifugation, the resultant cell pellet was resuspended in the culture medium and preincubated at 37 ℃ for 30 min for the subsequent experiments. All obtained pancreatic acinar cells were identified under a transmission electron microscope.

Cell viability assay

The cells were incubated with various concentrations of sodium taurocholate (final concentrations of 1, 2, 4, 6, 8, and 10 mmol/L) for 10, 20, 30, 40, and 50 min. The cell viability was determined using an MTT assay[19] to identify an appropriate concentration of sodium taurocholate for subsequent cell experiments.

The pancreatic acinar cells were divided into three groups, including a control group, an SAP model group, and a CEB group. The effects of CEB on the cell viability were determined using an MTT assay. The cells were preincubated with or without CEB (final concentration of 3.3 μmol/L) for 10 min prior to sodium taurocholate (final concentration of 8 mM) irritation. After an additional 30 min incubation with sodium taurocholate, the cell viability was measured using an MTT assay. The normal cells were treated with different concentrations of CEB (1.65, 3.3, 6.6, 13.2, 26.4, 52.8, and 105.6 μmol/L), and an MTT assay was performed. All experiments were performed in triplicate and repeated at least three times.

The cells were preincubated with various concentrations of CEB (final concentrations of 1.65, 3.3, 6.6, 13.2, 26.4, 52.8, and 105.6 μmol/L) for 30 min. The optical density value was detected using an MTT assay to assess the reliability of CEB at the cellular level.

Cell ultrastructural observation

The cells were pre-treated with or without CEB (final concentration of 3.3 μmol/L) for 10 min. Sodium taurocholate (1 mmol/L) was added to the cells as a stimulus for 1 min, immediately followed by washing with the PBS buffer, pelleting by centrifugation at 200 *g* for 1 min, and fixing using 2.5% prechilled glutaraldehyde at 4 ℃ for 24 h. The samples were then immersed in 1% osmium tetroxide at room temperature for 2 h and then uranyl acetate for the same period. The cells were dehydrated in serial solutions of ethanol and acetone before being embedded in epoxy resins. Then, 50-nm ultrathin sections were stained with lead citrate and mounted on 200-mesh copper grids. The cell ultrastructure was observed under a Hitachi H-7650 (Tokyo) transmission electron microscope.

Intracellular free Ca^{2+} measurement

Isolated pancreatic acinar cells were pre-treated with or without CEB (final concentration of 3.3 μmol/L) for 10 min. The cell suspension was washed once with PBS and incubated with fluo-3 AM (5 μmol/L, Invitrogen) for 30 min in the dark, followed by three washes with PBS. Then, 2 mL of PBS were added to the cells and incubated for an additional 30 min. Sodium taurocholate (1 mmol/L) was added to the cells as a stimulus. The cells were imaged on a live cell imaging system to assess the cytosolic Ca^{2+} concentration using fluo-3 AM (488 nm) immediately after the stimulus was applied, and the recording time was maintained at 60 s. The fluorescence intensity represented the relative intracellular Ca^{2+} concentration.

Detection of IP_3R expression

The cells were stimulated with sodium taurocholate as described above, immediately followed by washing with PBS and pelleting by centrifugation at 200 *g* for 1 min. Total RNA and protein were purified from the acinar cells according to the manufacturer's instructions (Ambion, Pierce). The protein and mRNA levels of IP_3R were quantified by RT-PCR and Western blot, respectively, as previously described[20].

Statistical analysis

The assays were performed in triplicate. All data are expressed as mean ± SD. Statistical comparisons were performed using *t*-tests or analysis of variance with SPSS 19.0 (SPSS Inc.). A *P*-value < 0.05 was considered statistically significant.

RESULTS

CEB protects against pancreatic injury in SAP rats

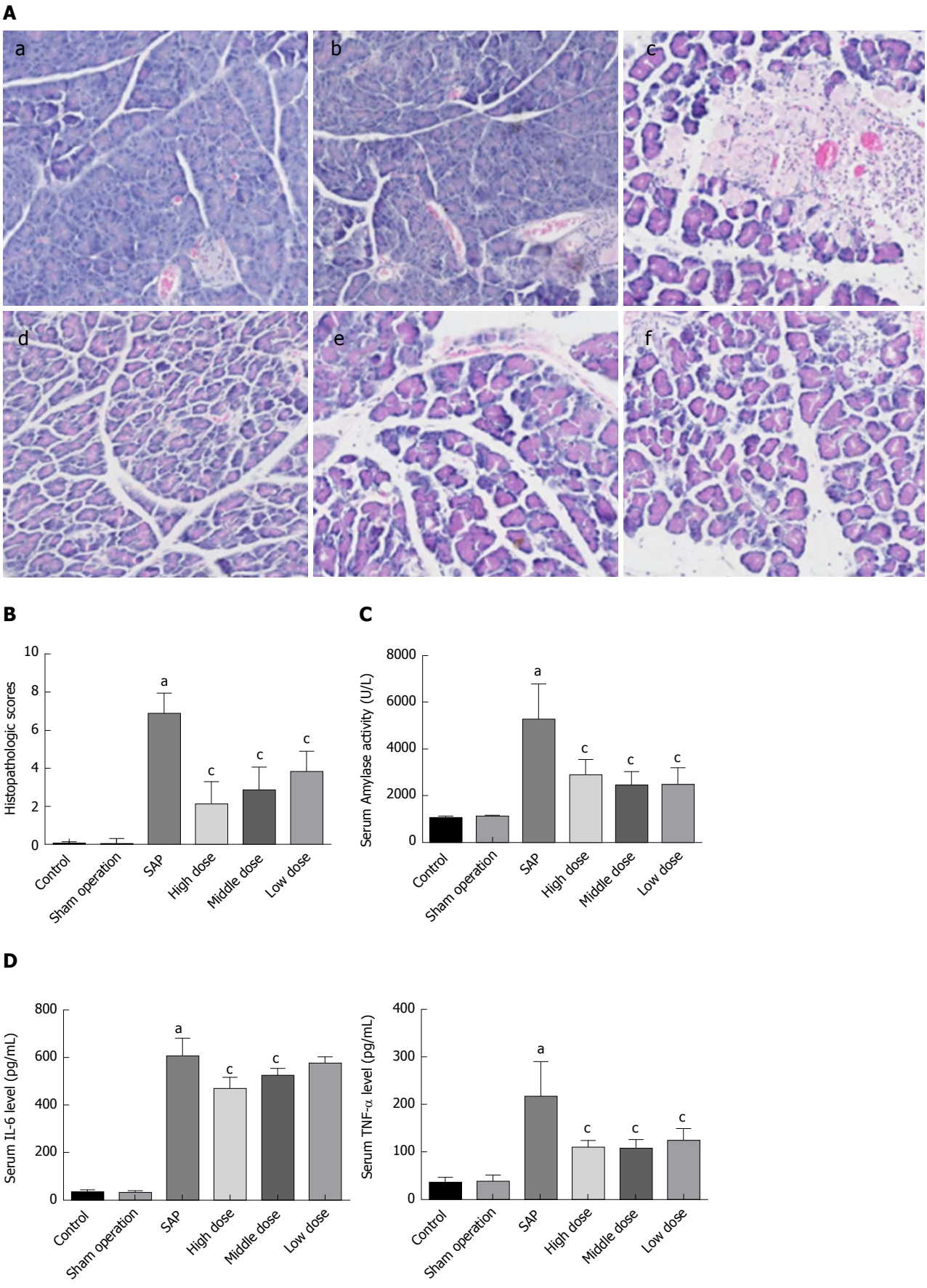
The SO group showed an entirely normal acinar architecture. The SAP group demonstrated pancreatic injury characterized by gross tissue oedema, sublobular haemorrhage, cell lysis associated with obvious neutrophil infiltration, and parenchymal necrosis. Treatment with CEB ameliorated pancreatic injury and decreased the histopathology score (Figure 1A and B, $P < 0.05$).

CEB alleviates amylase activity and inflammatory response in SAP rats

Serum amylase activity and levels of IL-6, TNF-α, and IL-10 in the SAP group were significantly increased compared to those in the SO group. Treatment with CEB decreased serum amylase activity and TNF-α level but increased the level of IL-10 at all the three doses (Figure 1C and D, $P < 0.05$). CEB decreased serum IL-6 level in SAP rats at high and middle doses (Figure 1D, $P < 0.05$). There was also a trend towards decreased IL-6 level at low dose; however, it did not reach statistical significance.

CEB increases the viability of sodium taurocholate-stimulated acinar cells

Different concentrations of sodium taurocholate induced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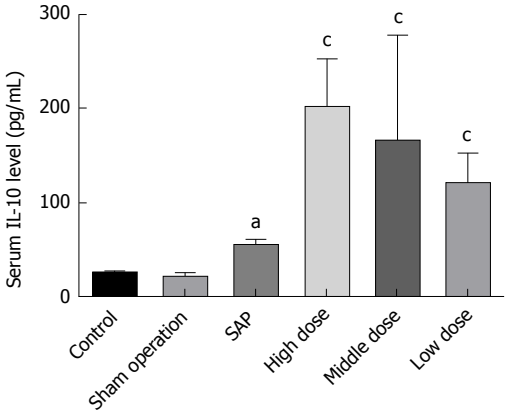

Figure 1 Therapeutic effects of combined use of emodin and baicalein on severe acute pancreatitis rats. Animals received CEB or an equal amount of saline injection after the SAP induction. CEB at high, middle, and low doses was administered immediately after the SAP induction. Animals were sacrificed 12 h after the SAP induction and were assessed for (A) representative images of pancreatic histopathology in all groups (H&E, ×100). (a) Control group; (b) SO group; (c) SAP group; (d) CEB high-dose group; (e) CEB middle-dose group; (f) CEB low-dose group. B: Histopathology score evaluation; C: Serum amylase; D: Serum cytokines. The data represent the mean ± SD. $^{a}P < 0.05$ *vs* the control group; $^{c}P < 0.05$ *vs* the SAP group.

Table 2 The duration of cells sustained at high calcium concentration

Group	Duration of cells sustained at high calcium concentration (s)
Control	0 ± 0.03
SAP	30 ± 1.77[a]
CEB	5 ± 1.80[c]

$^{a}P < 0.05$ *vs* control group; $^{c}P < 0.05$ *vs* SAP group. SAP: Severe acute pancreatitis; CEB: Combined use of emodin and baicalein.

a decrease in cell viability (Figure 2A, $P < 0.05$). Cell viability decreased in a dose- and time- dependent manner after sodium taurocholate treatment. Compared to the control group, cell viability was decreased in the SAP group. Pretreatment with CEB could increase the viability of cells treated with 8 mmol/L sodium taurocholate and showed dose-dependent protective effects at 1.65-3.3 μmol/L (Figure 2B, $P < 0.05$). Moreover, CEB alone had no adverse effect on the normal cells at either low or high concentration (Figure 2C, $P > 0.05$).

CEB alleviates sodium taurocholate-induced intracellular vacuole formation

The normal cells showed characteristics that are typical of secretory polarized cells, including slick plasmalemma and a roundish nucleus in the basal region of the cell. The rough endoplasmic reticulum (ER) was abundant and arranged in the parallel cisternae in the basal region, and numerous ribosomes were present on the membrane of the ER. Many zymogen granules (ZGs) were observed in the apical region of the cell. The mitochondria exhibited a spherical shape, and well-developed cristae were observed in the basal region of the cell (Figure 2D, a and d). In the SAP group, after the 1-min stimulation with 1 mmol/L sodium taurocholate, major changes were observed in the cellular ultrastructure, including the rupture of the plasmalemma, formation of abnormal vacuoles in the cytoplasm, dilatation and degranulation of the ER, lysis of the membranes of the rough ER, and a marked reduction in the density of the normal ZGs (Figure 2D, b and e). In cells that were preincubated with 3.3 μmol/L CEB for 10 min, the vacuolization, cytolysis, and degranulation of the ER were significantly alleviated (Figure 2D, c and f).

CEB decreases cytosolic Ca^{2+} concentration and down-regulates expression of IP_3R

In the control group, there was no change in the cell fluorescence intensity over time (Figure 3A and B, a). In the SAP group, the cell fluorescence intensity immediately increased within 2 s of adding the irritant sodium taurocholate to the cells compared with that before the stimulation. The cell fluorescence intensity lasted for 30 s at a high Ca^{2+} concentration and then began to decline (Figure 3A and B, b). In the CEB group, the cell fluorescence intensity increased within the first 2 s after the sodium taurocholate suscitation and was sustained by the high calcium concentration for 5 s before beginning to decline (Figure 3A and B, c). Thus, the duration during which the cells survived in a high Ca^{2+} plateau decreased in the CEB preincubated cells compared to that in the cells that were not preincubated with CEB (Table 2, $P < 0.05$).

Compared with the control group, the three isoforms of IP_3R mRNA and IP_3R protein were increased in the SAP group. CEB preincubation caused a significant decrease in the mRNA expression of the three IP_3R isoforms (Figure 4A, $P < 0.05$). In the presence of CEB, there was also a trend towards decreased IP_3R protein; however, it did not reach statistical significance (Figure

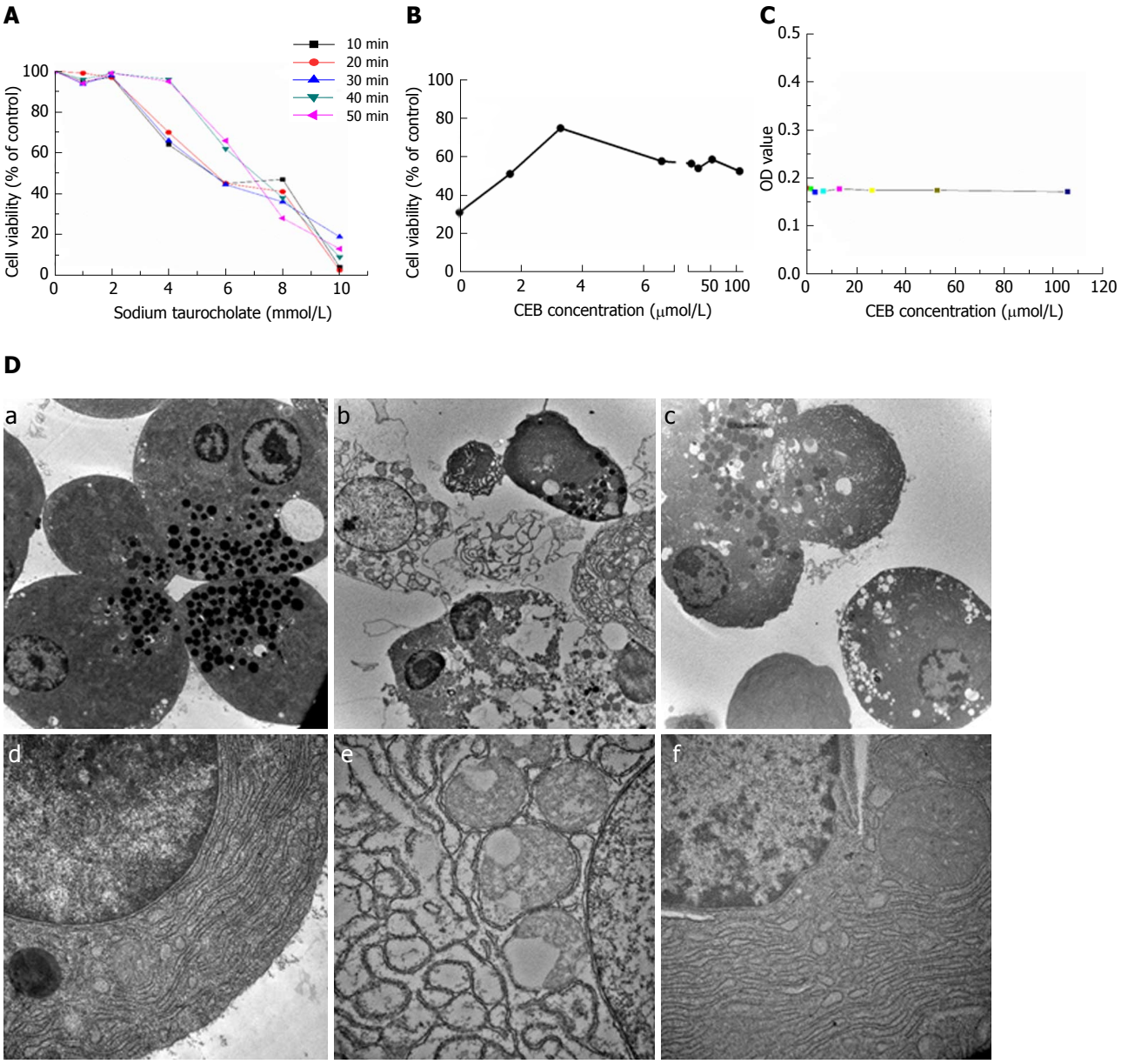

Figure 2 CEB alleviates cell injury in pancreatic acinar cells. A: The cell viability decreased in a concentration- and time-dependent manner after sodium taurocholate treatment. B: CEB pretreatment increased the viability of cells treated with 8 mM sodium taurocholate and showed dose-dependent protective effects at 1.65-3.3 μmol/L. C: CEB alone had no adverse effect on normal pancreatic acinar cells at either low or high concentration, which suggested its reliability at the cellular level. D: Ultrastructure of pancreatic acinar cells. (a and d) Normal acinar cells showed ZGs concentrated in the apical pole of the cell, slick plasmalemma, and a roundish nucleus in the basal region of the cell. The rough ER was abundant and arranged in the parallel cisternae in the basal region, and numerous ribosomes were present on the membrane of the ER. (b and e) Acinar cells after a 1-min stimulation with sodium taurocholate. Plasmalemma was ruptured, and vacuoles accumulated in the cytoplasm. The ER was dilated and degranulated with lytic membranes. The ZG density also showed a marked reduction. (c and f) Acinar cells preincubated with CEB for 10 min. Vacuolization, cytolysis, and degranulation of ER were significantly alleviated (a-c, ×5000; d-f, ×30000).

4B and C, $P > 0.05$).

DISCUSSION

The famous TCM Qingyi decoction has been shown to have clinical benefits in the treatment of SAP[5,7]. However, the challenges associated with its oral administration, complex components, and difficult-to-control quality limit its application and formulation. We refined Qingyi decoction and devised a compound formula with independent intellectual property rights, comprising emodin and baicalein, which we call CEB[13,14]. The components of CEB are the monomers emodin and baicalein, and the properties of CEB include clear configuration, controllable quality, and therapeutic effects following an intravenous injection in SAP rats; thus, CEB could be further developed for the treatment of patients with SAP and is worth further investigation. Our previous study has shown that the combination of emodin and baicalein exhibits good efficacy in SAP rats[13,15]. However, the specific mechanism remains unknown.

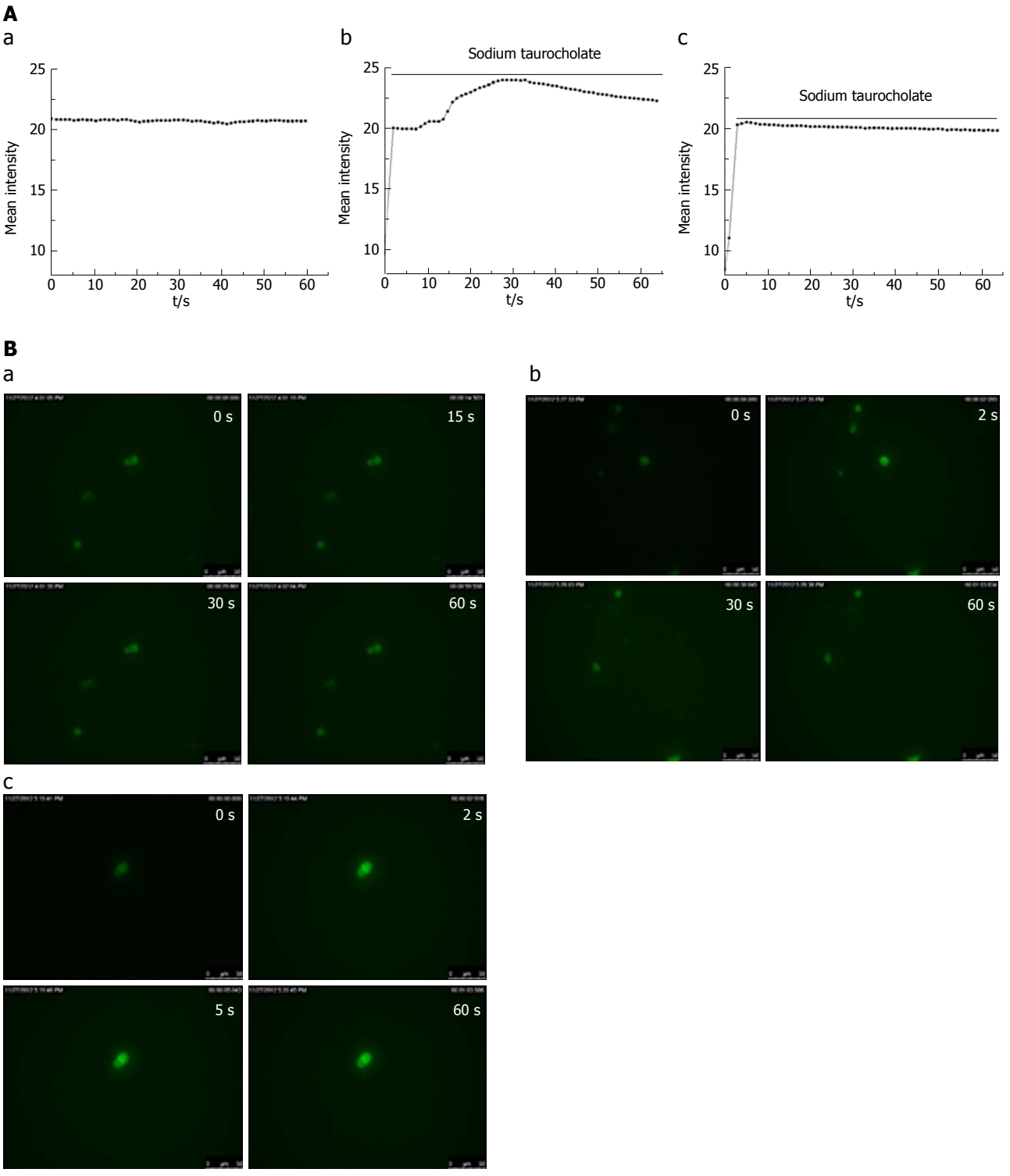

Figure 3 CEB inhibits sodium taurocholate-induced Ca^{2+} overload in isolated pancreatic acinar cells. Cell fluorescence intensity represents the cell calcium concentration. A: Dynamic change in the calcium concentration induced by sodium taurocholate was inhibited by CEB: (a) Unaltered fluorescence intensity over time in the control group. (b) Cell fluorescence intensity was strengthened immediately within 2 s of the sodium taurocholate irritation and lasted at a high calcium concentration for 30 s, followed by a decrease in the SAP group. (c) Cell fluorescence intensity was increased within the first 2 s after the sodium taurocholate suscitation, was sustained at a high calcium concentration for 5 s, and then began to decrease in the CEB group. B: Cell images at representative time points. (a) Control group. (b) SAP group. (c) CEB group.

The pathogenesis of SAP remains poorly understood[21]. One hypothesis posits that inflammatory reactions are crucial for the pathogenesis of SAP[22]. Local inflammatory reactions in the pancreas lead to systemic inflammatory response syndrome (SIRS) and multiorgan failure (MOF), which is believed to be the primary cause of mortality[23]. TNF-α and IL-6 are known principal pro-inflammatory cytokines that

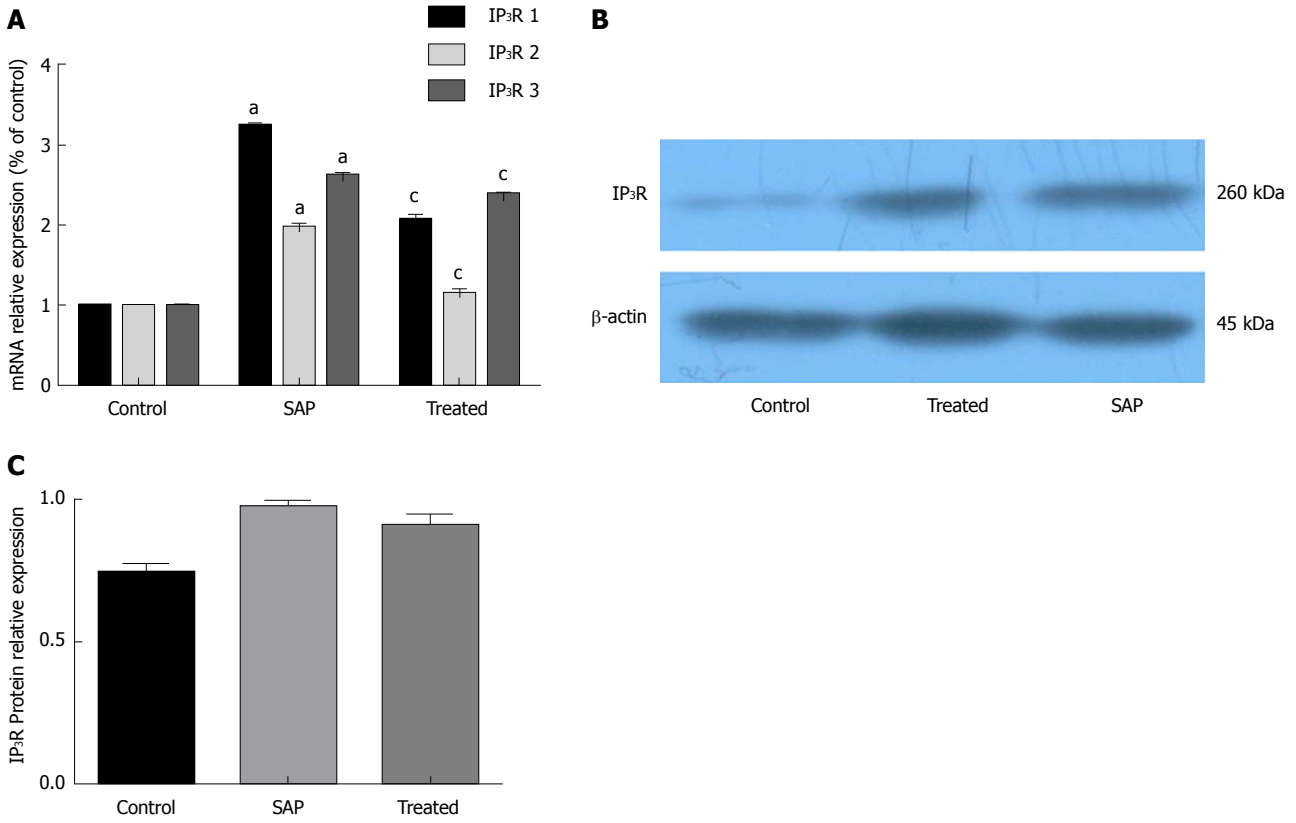

Figure 4 CEB decreases the expression of IP$_3$R in pancreatic acinar cells. A: Effects of CEB on the mRNA expression of three isoforms of IP$_3$R ($P < 0.05$). B and C: CEB caused a reduced trend of IP$_3$R protein expression but showed no significant difference ($P > 0.05$). $^{a}P < 0.05$ *vs* the control group; $^{c}P < 0.05$ *vs* the SAP group.

participate in the initiation and progression of SAP and have been identified as the key cytokines mediating pancreatitis-associated lung injury[11]. IL-10 has important anti-inflammatory activities and is strongly associated with the severity of SAP[24]. IL-10 is believed to play a protective role in SAP. In our study, we similarly found that serum levels of IL-6, TNF-α, and IL-10 were considerably higher in the SAP group than in the SO group. CEB reduced the pro-inflammatory cytokines IL-6 and TNF-α and increased the anti-inflammatory cytokine IL-10 after the induction of SAP.

The pathological diagnosis is a significant index for evaluating the severity of SAP. Amylase activity is an enzymatic marker, and the serum amylase test is routinely performed for the clinical diagnosis of SAP. In this study, CEB significantly alleviated pancreatic injury and decreased pancreatic histopathology score in the SAP model. Therefore, we conclude that CEB can down-regulate the pro-inflammatory cytokines IL-6 and TNF-α, up-regulate the anti-inflammatory cytokine, and decrease the serum amylase activity, thus reducing the pathologic score and ameliorating pancreatic injury. Therefore, the therapeutic effect of CEB on SAP warrants further study.

Ca^{2+} overload is the earliest crucial event in the pathogenesis of many diseases[25]. In pancreatic acinar cells, pathological Ca^{2+} overload is a critical trigger that initiates cell injury due to sustained global Ca^{2+} elevations, resulting in enzyme activation, vacuolization, and necrosis, all of which are crucial for the development of pancreatitis[26]. Emodin can inhibit pancreatic enzyme levels and calcium channels[27]. Baicalein has been reported to have antibacterial, antiviral, anti-inflammatory, and calcium blocker properties in cardiomyocytes[11,28]. However, whether CEB can inhibit Ca^{2+} overload during the development of SAP remains unclear. We used pancreatic acinar cells prepared from healthy rats that were induced by sodium taurocholate as an SAP model at the cellular level and observed the effect of CEB. In our study, sodium taurocholate exerted a cellular Ca^{2+} overload, leading to decreased cell viability, intracellular vacuole formation, and cellular injury. However, CEB inverted this impairment and did not show a cytotoxic effect in normal acinar cells, revealing its safety and hinting at the mechanism of CEB action.

IP$_3$R is a principal Ca^{2+}-releasing channel that is concentrated in the ER membrane, and the opening of this channel is coordinated by the binding of IP$_3$ and the activation of gated Ca^{2+} channels that release intracellularly stored Ca^{2+} from the ER to regulate the cellular Ca^{2+} environment[29]. In our study, at the cellular level, CEB decreased the sodium taurocholate-induced intracellular Ca^{2+} and IP$_3$R mRNA expression in isolated rat pancreatic acinar cells. Thus, CEB exerts effects on the inhibition of the acinar cell calcium overload, possibly representing its underlying mechanism in SAP cells. The protein expression changes required a

certain amount of time. To detect the calcium changes, we performed the observations 60 s after the sodium taurocholate stimulation, which may explain why the IP_3R protein expression showed a slight decline and no statistical significance in the CEB group compared with that in the SAP group.

In summary, CEB was found to act on the key aspects of SAP pathogenesis and resulted in the regulation of inflammatory factors and the inhibition of calcium overload, which underlie this synergy. These results at the cellular and organism levels reflect a preliminary mechanism of CEB in SAP and suggest potential clinical benefits. Although research on CEB remains at the preclinical level, and data on its use in humans are lacking, CEB may be a potential future treatment for SAP, suggesting that further studies are needed.

ARTICLE HIGHLIGHTS

Research background

Severe acute pancreatitis (SAP) is a debilitating disease with significant morbidity and mortality. Somatostatin analogue octreotide is the most frequently used drug but not all patients can afford its high cost and short half-life *in vivo*. Guided by the theories of TCM and modern medicine and the compatibility of herbs, we refined the classic prescription Qingyi decoction and combined emodin and baicalein into "Compound Emodin and Baicalein" (CEB, patent No. ZL200310105814.3). This research may provide a novel, safe, effective, and inexpensive drug for SAP treatment in the future.

Research motivation

The aim of this study was to explicate the effect and mechanism of CEB in SAP therapy and provides potential clinical benefits for the future treatment of SAP.

Research objectives

The SAP rats and the isolated pancreatic acinar cells were the main objectives in this study. Meanwhile, they also served as the main objectives of research on SAP at the cellular and organism levels.

Research methods

Retrograde infusion of 5% sodium taurocholate into the pancreatic duct is the frequently used method for SAP model induction. Serum cytokine levels were determined using enzyme-linked immunosorbent assay. Tissue section were stained using haematoxylin and eosin (H&E) for histopathologic detection. Pancreatic acinar cells were isolated using collagenase digestion and the cell viability was determined using an MTT assay. Cell ultrastructure was observed using a transmission electron microscope. Intracellular Ca^{2+} concentration was determined using a live cell imaging system. The protein and mRNA levels of IP_3R were quantified by RT-PCR and Western blot, respectively.

Research results

The results showed that CEB can regulate inflammatory factors, increase cell viability, and inhibit calcium overload, which underlie a preliminary mechanism of CEB in SAP. Although research on CEB remains at the preclinical level, and data on its use in humans are lacking, CEB may be a potential future treatment for SAP, suggesting that further studies are needed.

Research conclusions

Emodin and baicalein display synergistic effects on SAP rats. CEB acts on the key aspects of SAP pathogenesis and regulates of inflammatory factors in SAP rats. CEB suppresses IP3R-mediated pancreatic acinar Ca2+ overload *via* inhibiting IP3R mRNA expression in pancreatic acinar cells. As a non-toxic natural product, CEB is the first to show a therapeutic effect at the cellular and organism levels in SAP. CEB is promising for the treatment of patients with SAP.

Research perspectives

From this study, the characteristics of abundance, low cost, and safety of traditional Chinese medicine in SAP therapy should catch our attention. We think that the potential of traditional Chinese medicine in SAP therapy will be the direction of the future research. In our opinion, the SAP animal model and the isolated pancreatic acinar cells will always be the main avenues in this study.

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ORIGINAL ARTICLE

Case Control Study

Increased intestinal mucosal leptin levels in patients with diarrhea-predominant irritable bowel syndrome

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Abstract

AIM

To measure the leptin levels in patients with diarrhea-predominant irritable bowel syndrome (IBS-D) and analyze the relationship of leptin with clinical features, visceral sensitivity, mast cells, and nerve fibers.

METHODS

Forty-two patients with IBS-D fulfilling the Rome Ⅲ criteria and 20 age- and sex-matched healthy controls underwent clinical and psychological evaluations using validated questionnaires (including IBS Symptom Severity Scale, IBS-specific Quality of Life, Hamilton Anxiety Scale, and Hamilton Depression Scale), along with colonoscopy, colonic mucosal biopsy, and visceral sensitivity testing. Serum leptin levels were assayed using enzyme-linked immunosorbent assay. Mucosal leptin expression and localization were evaluated using immunohistochemistry and immunofluorescence.

Mucosal leptin mRNA levels were quantified using quantitative real-time reverse transcription polymerase chain reaction. Mast cell counts and activation rates were investigated by toluidine blue staining. Correlation analyses between these parameters were performed.

RESULTS

There were no statistically significant differences in age, gender, or body mass index between the IBS-D group and the control group. The median IBS Symptom Severity Scale score in the IBS-D group was 225.0 (range, 100-475). IBS-D patients had significantly increased anxiety [IBS-D: median, 6.5; interquartile range (IQR), 3.3; control: median, 2.0; IQR, 2.0; $P < 0.001$] and depression (IBS-D: median, 7.0; IQR, 3.0; control: median, 3.0; IQR, 2.0; $P < 0.001$) scores. IBS-D patients had significantly lower first sensation threshold (IBS-D: median, 50.6; IQR, 25.9; control: median, 80.5; IQR, 18.6; $P < 0.001$), defecation sensation threshold (IBS-D: median, 91.5; IQR, 29.3; control: median, 155.0; IQR, 21.1; $P < 0.001$) and maximum tolerable threshold (IBS-D: median, 163.2; IQR, 71.2; control: median, 226.2; IQR, 39.3; $P < 0.001$). Mucosal leptin expression, as reflected by integrated optical density (IBS-D: median, 4424.71; IQR, 4533.63; control: median, 933.65; IQR, 888.10; $P < 0.001$), leptin mRNA expression (IBS-D: median, 1.1226; IQR, 1.6351; control: median, 0.8947; IQR, 0.4595; $P = 0.009$), and mast cell activation rate (IBS-D: median, 71.2%; IQR, 12.9%; control group: median, 59.4%; IQR, 18.88%; $P < 0.001$) were significantly increased in IBS-D patients. The colocalization of leptin and leptin receptors was observed on mast cells and PGP9.5-positive nerve fibers in the intestinal mucosa. Also, leptin expression was positively correlated with anxiety, depression, and the mast cell activation rate, but negatively correlated with the defecation sensation threshold and the maximum tolerance threshold during visceral sensitivity testing (adjusted $P < 0.0038$).

CONCLUSION

Increased levels of mucosal leptin may interact with mast cells and the nervous system to contribute to the pathogenesis of IBS-D.

Key words: Leptin; Irritable bowel syndrome; Mast cells; Diarrhea; Visceral hypersensitivity

Core tip: Leptin is an important cytokine that exerts significant biological effects on gastrointestinal function and immune system modulation. We found that diarrhea-predominant irritable bowel syndrome (IBS-D) patients had significantly increased psychological symptoms, visceral hypersensitivity, mucosal leptin expression, leptin mRNA expression, and mast cell activation rate. Additionally, leptin expression was positively correlated with anxiety, depression, and the mast cell activation rate, but negatively correlated with the defecation sensation threshold and maximum tolerance threshold during visceral sensitivity testing. Increased levels of mucosal leptin may interact with mast cells and the nervous system to contribute to the pathogenesis of IBS-D.

Liu DR, Xu XJ, Yao SK. Increased intestinal mucosal leptin levels in patients with diarrhea-predominant irritable bowel syndrome. *World J Gastroenterol* 2018; 24(1): 46-57 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i1/46.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i1.46

INTRODUCTION

Irritable bowel syndrome (IBS) is a commonly diagnosed functional gastrointestinal disease, with a prevalence rate of 5%-10% in most European countries, the United States, and China[1]. IBS is a typically recurrent disease characterized by abdominal pain or discomfort, stool irregularities, and bloating[2]. Symptoms may worsen over time and significantly impact patients' quality of life and work productivity[3]. A major subtype of IBS is IBS with diarrhea (IBS-D), which accounts for 46% of all IBS cases[4].

The pathogenesis of IBS-D is complex and poorly understood. Recent studies of the pathophysiology of IBS-D have focused on molecular mechanisms and have suggested that the levels of luminal and mucosal cytokines originating from the gastrointestinal tract may be altered in the gut of IBS-D patients. These alterations could result in dysregulation of gastrointestinal secretion and motility and an increase in visceral hypersensitivity. These cytokines can also affect the peripheral and central nervous systems and disrupt communication between the brain and gut[5].

Leptin is a 16 kDa non-glycosylated peptide hormone belonging to the type I cytokine superfamily[6]. It is produced predominantly by mature adipocytes, but also by gastric and colonic epithelial cells, immune cells, placental trophoblasts, amniotic cells, chondrocytes, and synoviocytes[7]. When bound to receptor sites, leptin not only exerts significant biological effects, such as appetite control, by signaling satiety and increasing energy expenditure, but also modulates the immune system[8] and gastrointestinal function[9,10].

The number of mucosal mast cells has been reported to be increased in IBS-D patients[11], and mast cell number often correlates with symptoms including abdominal pain[12] and bloating[13]. Mast cells are key to the induction and maintenance of low-grade immune activation in IBS-D patients[14]. Gastrointestinal mast cells can express leptin and leptin receptors, indicating the modulatory effects of leptin on mast cells[15].

The enteric nervous system (ENS) is important in regulating gut motility, secretion, and nutrient absorption. Leptin has been shown to affect the ENS[16,17] and

sensory afferent neurons[18]. Taken together, leptin may be involved in the regulation of both local gastrointestinal functions and the brain-gut axis.

Few studies have specifically addressed the role of leptin in the pathogenesis of IBS[19-21]. Therefore, the present study measured leptin expression in both the serum and intestinal mucosa of patients with IBS-D, and then analyzed the relationship of leptin with the clinical features, visceral sensitivity, the number and activation rate of mast cells, and nerve fibers in these patients

MATERIALS AND METHODS

Subjects

We recruited 42 IBS-D patients (15 women and 27 men; mean age, 29.4 years; age range, 22-40 years) and 20 healthy controls (8 women and 12 men; mean age, 28.9 years; age range, 20-38 years). All patients were treated at the gastroenterology department of the China-Japan Friendship Hospital from January 2016 to July 2016. IBS-D was diagnosed according to the Rome Ⅲ criteria. Controls were recruited through public advertisement or from asymptomatic patients undergoing colonoscopy for colorectal cancer screening or polyposis follow-up. Exclusion criteria included the use of anti-spasmodics, analgesics, antibiotics, nonsteroidal anti-inflammatory drugs, corticosteroids, mast cell stabilizers, and anti-depressants or a history of organic diseases (documented by patient history, appropriate consultation, and laboratory testing), celiac disease (documented by celiac serology), allergic disease, major abdominal surgery, and severe psychiatric disorders.

All participants underwent venous blood sampling and colonoscopy. Before colonoscopy, all subjects underwent a standard bowel preparation in the form of 3 L of dissolved polyethylene glycol electrolyte powder (Fortrans, Beaufour Ipsen Industrie, Dreux, France). Four biopsies were obtained in each subject from the rectosigmoid junction in order to standardize the site of sampling. Two biopsy specimens were used for routine hematoxylin and eosin (H and E) staining, mast cell staining, and immunohistochemistry. The other two biopsy specimens were used for quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR).

IBS-D patients and control patients both gave informed consent in a written form before entry into the study. The study protocol was approved by the China-Japan Friendship Hospital Ethics Committee (No. 2015-33) and the study was conducted in accordance with the Declaration of Helsinki.

Measures

Questionnaires: Clinical status was evaluated with the use of validated questionnaires. The IBS Symptom Severity Scale[22,23], a 5-item self-reporting questionnaire designed to measure disease severity, was given to each participant to complete. Questionnaire items included information regarding abdominal pain, bloating, satisfaction with bowel habits, and overall interference with quality of life. The total score of the questionnaire ranges from 0 to 500.

The IBS-specific Quality of Life questionnaire[24,25] was used to evaluate changes in patients' quality of life. This scale evaluates 34 broad well-being factors based on variables including feelings of dysphoria, social interactions, body image, and health worries. The Hamilton Anxiety Scale[26,27] and Hamilton Depression Scale[27,28] were used to measure anxiety and depression, respectively.

Visceral sensitivity testing: Participants were placed in the left lateral decubitus position. After a digital rectal examination, a lubricated catheter with a latex balloon at the tip (Medical Measurement Systems, Enschede, the Netherlands) was inserted into the rectum such that the balloon was 8 cm proximal to the anal verge. Then, the balloon was inflated at a speed of 10 mL/5 s. During balloon inflation, feelings of initial perception, defecation urge, and discomfort/pain were reported by the participants. The balloon volumes were recorded at the first sensation threshold, defecating sensation threshold, and maximum tolerable threshold. All the tests were performed by the same investigator between 1 and 3 PM in a blinded fashion.

Serum leptin level: All fasting blood samples from both IBS-D patients and controls were collected between 11 AM and 1 PM to avoid diurnal variation. Whole blood was centrifuged immediately at 1151 *g* for 10 min, and stored at -80 ℃ until assay. Serum leptin levels were measured in duplicate using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Human Leptin Instant ELISA; Bender MedSystems GmbH, Vienna, Austria).

Histology, immunohistochemistry, and immunofluorescence: H and E sections from formalin-fixed and paraffin-embedded tissue samples were blindly assessed by independent observers. Mast cells were stained with toluidine blue. The slides were first soaked in 0.5% toluidine blue, and then differentiated with acetone. Afterwards, five 400 × magnification fields (field area, 0.237 mm^2) were chosen randomly and scanned. Mast cells were identified using light microscopy by their metachromatic cytoplasmic granules. Finally, mast cell degranulation was assessed based on unclear or irregular cell membranes and the presence of extruded secretory granules. The mean mast cell number per millimetre square of the mucosal area (/mm^2) was calculated. The percentage of degranulated mast cells (mast cell activation rate) was also calculated in each section (degranulated mast cells/the total number of mast cells × 100%).

Immunohistochemistry and immunofluorescence experiments were performed on paraffin-embedded, 4-mm-thick sections. The following primary antibodies

were used: rabbit polyclonal anti-leptin antibody (1:100; Abcam, Cambridge, United Kingdom), rabbit polyclonal anti-leptin receptor antibody (1:50; Abcam, Cambridge, United Kingdom), mouse monoclonal anti-mast cell tryptase antibody (1:50; Abcam, Cambridge, United Kingdom), and mouse monoclonal anti-PGP9.5 antibody (1:50; Abcam, Cambridge, United Kingdom).

For immunohistochemistry studies, the sections were first incubated with the primary antibody overnight. Next, they were incubated at room temperature with a universal secondary antibody (EnVision Detection Systems, Dako, Denmark) for 60 min and then visualized using diaminobenzidine. Finally, the nuclei were labeled by counterstaining the sections with Mayer' s hematoxylin. The quantification of immunoreactivity was performed by two operators in a blinded fashion. Three randomly selected fields from each section were scanned under a Nikon Eclipse 80i microscope (Nikon Instruments Co., Ltd., Tokyo, Japan). Photographs were taken with a Nikon DS-Ri1 camera (Nikon Instruments Co., Ltd., Tokyo, Japan). The images were analyzed with Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, United States). During the analysis, the average integrated option density (IOD) of positive staining substances of three non-overlapping fields chosen randomly was measured for the final analysis.

For immunofluorescence studies, the sections were prepared sequentially as follows: incubated with primary antibody (double labeling) overnight, rinsed with phosphate-buffered saline, and incubated at room temperature with goat anti-mouse IgG H and L (Alexa Fluor 647) (1/300; Abcam, Cambridge, United Kingdom) or goat anti-rabbit IgG H and L (Alexa Fluor® 488) (1/400; Abcam, Cambridge, United Kingdom) for 2 h. Specimens were examined by two operators in a blinded fashion using a Nikon Eclipse 90i microscope (Nikon Instruments Co., Ltd., Tokyo, Japan). Representative photomicrographs were taken using a Nikon DS-Ri1 camera.

Leptin gene expression analysis using qRT-PCR: The gene expression of leptin and the leptin receptor in the colonic mucosa was analyzed using qRT-PCR. Each supernatant was assayed in duplicate, and altogether three independent experiments were conducted. TRIzol reagent (Invitrogen Life Technologies, Waltham, MA, United States) was used to isolate total RNA. Then, the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, United States) was used to perform reverse transcription. Next, real-time PCR was carried out using the FastStart Universal SYBR Green Master Rox kit (Roche, Shanghai, China) in the StepOnePlus Real-Time PCR System (Applied Biosystems, Waltham, MA, United States). Finally, leptin and leptin receptor mRNA expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. The following primers were used for PCR amplification: forward, 5′-ACTTTGGTATCGTGGAAGGACTCAT-3′ and reverse, 5′-GTTTTTCTAGACGGCAGGTCAGG-3′ for GAPDH; forward, 5′-ACACGCAGTCAGTCTCCTCCAA-3′ and reverse, 5′-CTGGAAGGCATACTGGTGAGGA-3′ for leptin; and forward, 5′-GCCAACAGCCAAACTCAACG-3′ and reverse, 5′-GGTGGGCTGGACCAAGAAATC-3′ for leptin receptor. The results were first normalized to GAPDH expression using the $2^{-\Delta\Delta Ct}$ method[29]. Then, the leptin and leptin receptor mRNA levels in each sample are expressed as the fold-change relative to the average level of healthy controls, in whom the mean value was used as the reference and considered as 1.

Statistical analysis

All statistical analyses were performed using SPSS for Windows software, version 24.0 (SPSS Inc, Chicago, IL). All data are reported as values (mean ± SD) or medians [interquartile range (IQR)]. Independent sample *t*-tests or nonparametric Mann-Whitney *U*-tests were used to analyze quantitative data. The χ^2 test or Fisher's exact test were used to analyze qualitative data. Correlations between two parameters were performed using Spearman's correlation coefficient, followed by Bonferroni correction to adjust multiple comparisons, with a corrected significance level of 0.0038 (0.05/13). Two-tailed *P*-values < 0.05 were considered significant.

RESULTS

Demographic and clinical characteristics

There were no statistically significant differences in age, gender, or body mass index between the IBS-D group and the control group. The median IBS Symptom Severity Scale score in the IBS-D group was 225.0 (range, 100-475). There was no significant difference between male and female IBS-D patients in terms of IBS Symptom Severity Scale score (P = 0.053). The median duration of disease was 3.5 years (range, 1-13 years) in the IBS-D patients. The IBS-specific Quality of Life score was significantly lower in IBS-D patients (P < 0.001), and the differences in the scores of both the Hamilton Anxiety Scale and the Hamilton Depression Scale were also statistically significant between the IBS-D group and the control group (P < 0.001) (Table 1).

Visceral sensitivity test

The first sensation threshold, defecation sensation threshold, and maximum tolerable threshold were significantly lower in the IBS-D patients compared with those in the control group (P < 0.001) (Table 1).

Serum leptin

Serum leptin levels in the IBS-D patients (median, 1.28 ng/mL; IQR, 1.63 ng/mL) and healthy controls (median, 1.27 ng/mL; IQR, 0.83 ng/mL) were not

Table 1 Demographics, clinical characteristics, and visceral sensitivity of irritable bowel syndrome patients and healthy controls

	IBS-D	Controls	*P* value
Demographic and clinical feature			
N	42	20	NA
Age (yr)	29.4 ± 4.3	28.9 ± 5.1	0.673
Gender (male: female)	9:5	3:2	0.744
Body mass index (kg/m^2)	22.5 ± 2.5	22.1 ± 2.2	0.553
Duration of disease (yr)	3.5 (3.3)	NA	NA
Questionnaire			
IBS Symptom Severity Scale	225.0 (50.0)	NA	NA
IBS-specific Quality of Life	76.5 (17.3)	100.0 (1.1)	< 0.001
Hamilton Anxiety Scale	6.5 (3.3)	2.0 (2.0)	< 0.001
Hamilton Depression Scale	7.0 (3.0)	3.0 (2.0)	< 0.001
Visceral sensitivity			
First sensation threshold (mL)	50.6 (25.9)	80.5 (18.6)	< 0.001
Defecating sensation threshold (mL)	91.5 (29.3)	155.0 (21.1)	< 0.001
Maximum sensation threshold (mL)	163.2 (71.2)	226.2 (39.3)	< 0.001

The data are expressed as mean ± SD, or median (interquartile range). IBS-D: Irritable bowel syndrome; NA: Not applicable.

significantly different (P = 0.657).

Mucosal histology and mast cell staining

On H and E histology, all colonic mucosal biopsies were reported as normal (Figure 1A and B). Toluidine blue staining revealed that mast cells were often scattered in the lamina propria and submucosa (Figures 1C and D). The mast cell count was not significantly different between the two groups (IBS group: median, 9.62/mm^2; IQR, 1.61/mm^2 *vs* control group: median, 10.75/mm^2; IQR, 4.52/mm^2; P = 0.164) (Figure 1E). However, the percentage of degranulated mast cells was significantly increased in IBS-D patients (IBS group: median, 71.2%; IQR, 12.9% *vs* control group: median, 59.4%; IQR, 18.88%; P < 0.001) (Figure 1F).

Leptin and leptin receptor expression in the colonic mucosa

In the colonic mucosa, leptin and leptin receptor staining was mostly scattered in the epithelium and lamina propria (Figure 2A-D). The leptin protein level in mucosal biopsies was significantly increased in the IBS-D patients (IOD median, 4424.71; IQR, 4533.63) *vs* the control group (IOD median, 933.65; IQR, 888.10; P < 0.001) (Figure 2E). The leptin receptor expression was not significantly increased in the colonic mucosa obtained from IBS-D patients (12454.27 ± 6946.06) compared to controls (12508.07 ± 8088.46) (P = 0.979) (Figure 2F).

Colocalization of leptin and leptin receptors on mast cells and PGP9.5-positive nerve fibers

Leptin and tryptase double-labeling immunofluorescence experiments recorded colocalization of leptin and tryptase, indicating that mast cells were producers of leptin (Figure 3A and B). Moreover, leptin receptor and tryptase colocalization was found to exist in the colonic mucosa (Figure 4A and B). Double-staining experiments confirmed leptin receptor expression in PGP9.5-immunoreactive nerve fibers (Figure 4C and D).

Leptin and leptin receptor gene expression in the colonic mucosa

Leptin mRNA expression was significantly increased in IBS-D patients (median, 1.1226; IQR, 1.6351) *vs* that seen in the control group (median, 0.8947; IQR, 0.4595; P = 0.009). Similarly, leptin receptor gene expression was also significantly increased in IBS-D patients (median, 1.5491; IQR, 2.0721) *vs* that seen in the control group (median, 0.9062; IQR, 0.3850; P = 0.001).

Correlation analysis

In patients with IBS-D, the leptin protein expression level showed a significantly positive correlation with anxiety, depression, and mast cell activation rate, and a negative correlation with the defecation sensation threshold and maximum tolerable threshold (adjusted P < 0.0038). The leptin mRNA level was significantly positively correlated with IBS symptom severity, anxiety, and depression, and negatively correlated with the visceral sensation threshold (adjusted P < 0.0038) (Table 2).

DISCUSSION

The present study investigated the possible role of leptin in the pathogenesis of IBS-D. Biopsies from IBS-D patients revealed significant upregulation of both leptin protein and mRNA levels. The increased leptin protein or mRNA expression closely correlated with IBS symptom severity, anxiety, depression, and visceral sensation thresholds, which may provide a putative basis for the development of IBS symptoms. To our knowledge, this was the first study to preliminarily confirm that leptin may play a certain role in the pathophysiology of IBS-D.

Visceral hypersensitivity has been identified in 20%-90% of IBS patients and is defined as a low threshold for the perception of stimuli arising from the gut[30,31]. Mechanisms of visceral hypersensitivity are complex, including the sensitization of gut wall sensory endings, enhanced nociceptive information, reduced antinociceptive effects at the level of the spinal cord, and hypervigilance of the central nervous system[31]. In line with these mechanisms, the present study found that visceral sensation thresholds were significantly lower in IBS-D patients than those of healthy controls. Limitations regarding the method of rectal distention used were discussed in our previous study[32] and include: (1) the sensory threshold, reported by the subject, may be susceptible to a perceptual response bias[33]; (2) progressive rectal distention may lead to hypervigilance and alter the response of the participants; and (3) volume measurements, rather than balloon pressures recorded with a barostat, may

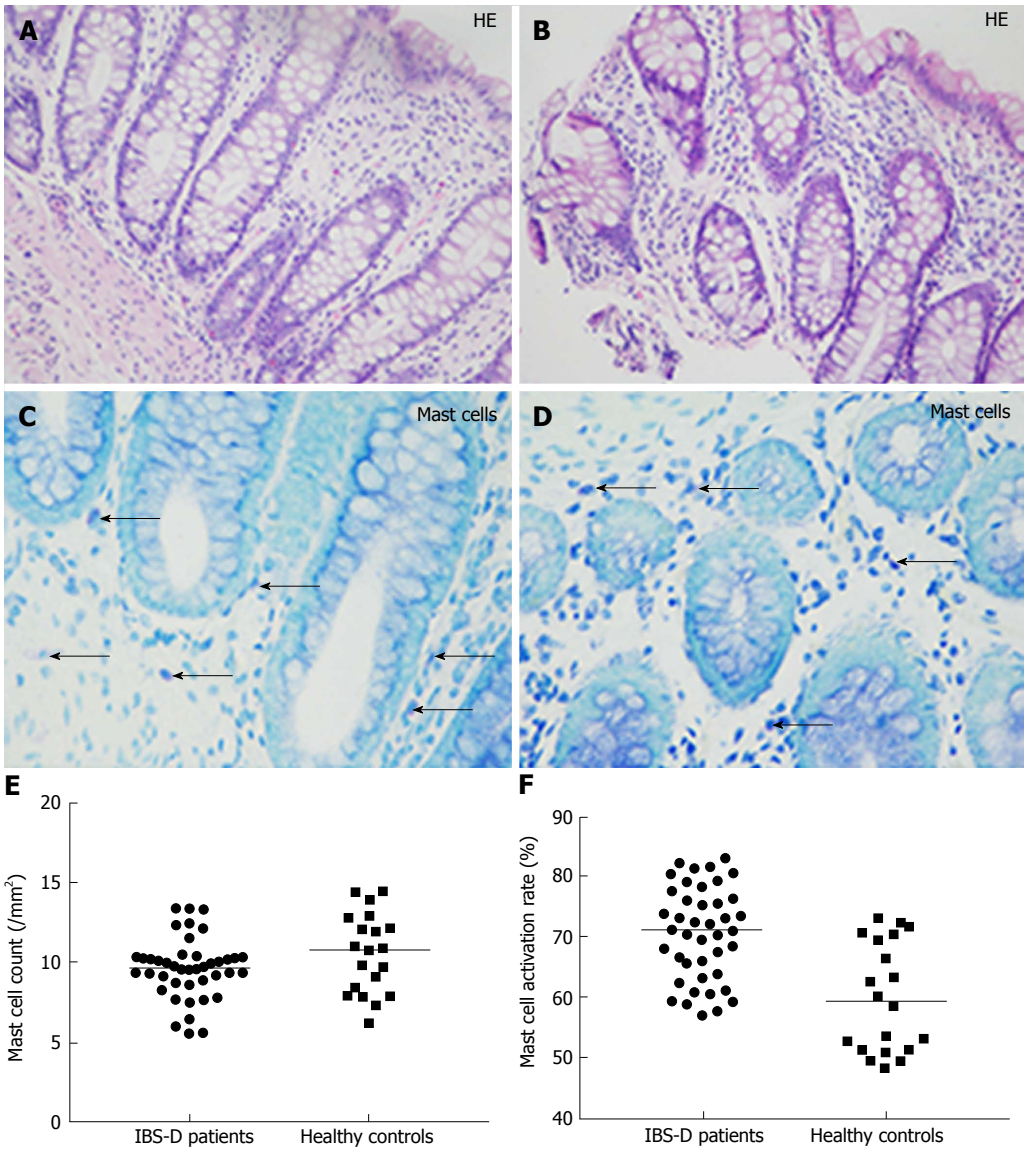

Figure 1 Intestinal mucosal histology (hematoxylin and eosin staining), mast cell toluidine blue staining, mast cell count (/mm^2), and mast cell activation rate (%) in irritable bowel syndrome patients and controls. A: Colonic mucosal histology of IBS-D patients (× 200); B: Colonic mucosal histology of healthy controls (× 200); C: Mast cell toluidine blue staining of IBS-D tissue (× 400, arrows represent mast cells); D: Mast cell toluidine blue staining of normal control tissue (× 400, arrows represent mast cells); E: Mast cell count was not significantly different between the two groups (P = 0.164); F: Mast cell activation rate significantly increased in IBS-D patients (P < 0.001). Line in the scatter plots means the median. IBS-D: Iirritable bowel syndrome with diarrhea.

not accurately reflect the biological properties of the rectum, leading to measurement errors[34]. In spite of these limitations, this method is convenient to perform and well-tolerated by patients. Furthermore, some studies have shown that volume thresholds are a valid measure of rectal sensory function[35-38].

Mast cell proliferation has been recognized as a common feature of IBS patients[39-41]. Some studies have suggested that the number of mast cells is increased in IBS patients[42,43], while other studies have shown that the density of mast cells is not significantly different in these patients[44-46]. Additional studies have shown that the degranulation or activation rate of mast cells, rather than the number of mast cells, best reflects the severity of IBS in patients[47]. In IBS patients, activated mast cells release mediators such as histamine, serotonin, and bradykinin[41]. These mediators contribute to the development of major IBS-D symptoms through the modulation of visceral sensation, gastrointestinal motility, intestinal immune function, and epithelial permeability and secretion[41]. In the present study, the mast cell activation rate was found to be significantly higher in IBS-D patients, while the mast cell count was not significantly different from that seen in the control group, indicating that activated mast cells are important in the pathophysiology of IBS-D.

Few studies have specifically analyzed serum leptin levels in IBS patients[19-21], and the results of these studies have been inconsistent. In the present study, serum leptin levels in patients with IBS-D were not significantly different from those seen in healthy controls. However, both leptin and leptin receptor

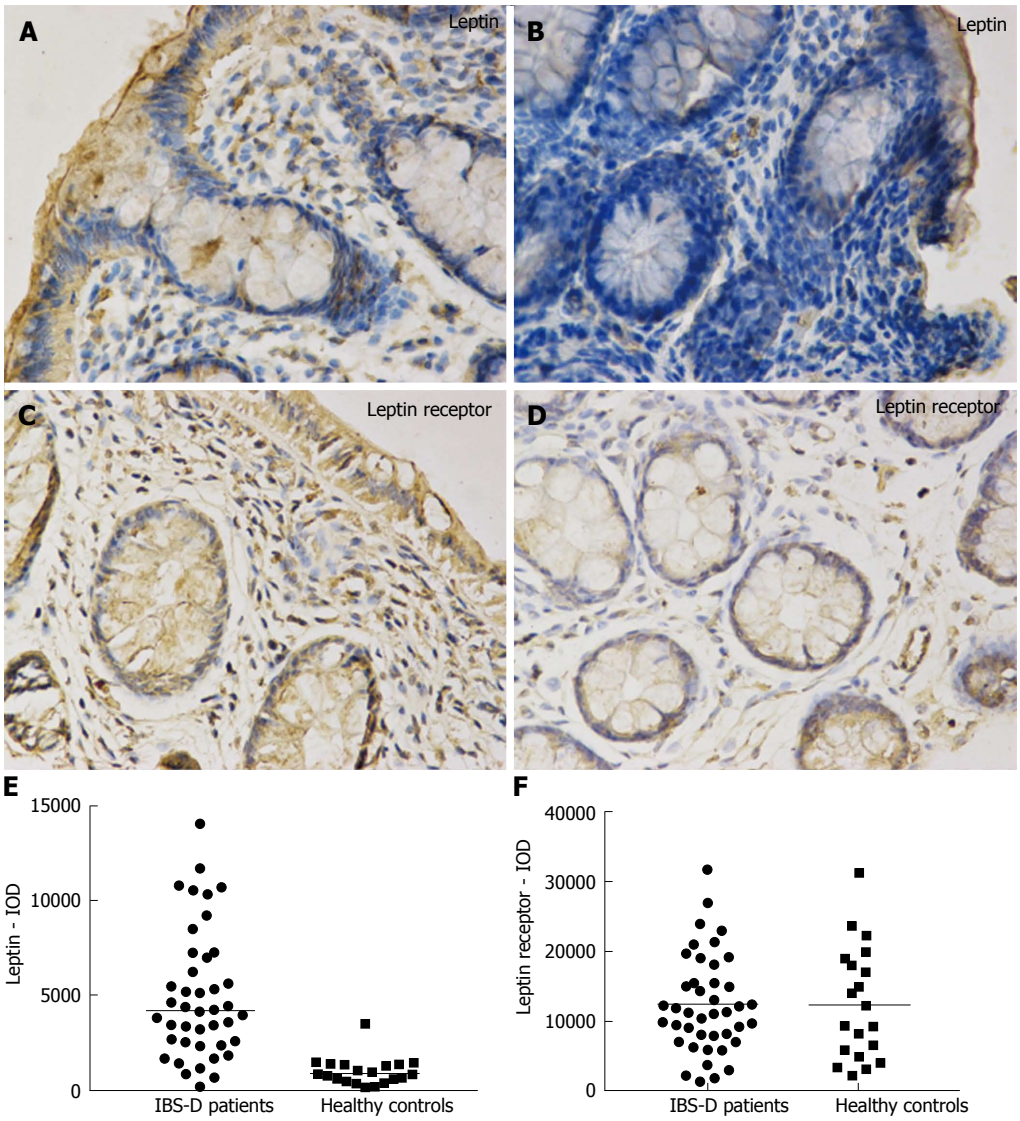

Figure 2 Immunohistochemical staining for leptin and leptin receptor in irritable bowel syndrome patients and healthy controls. A: Colonic mucosal leptin expression in IBS-D patients (× 400); B: Colonic mucosal leptin expression in healthy controls (× 400); C: Colonic mucosal leptin receptor expression in IBS-D patients (× 400); D: Colonic mucosal leptin receptor expression in healthy controls (× 400); E: Colonic mucosal leptin immunoreactivity significantly increased in IBS-D patients than in the healthy controls; F: Colonic mucosal leptin receptor immunoreactivity did not increase significantly in IBS-D patients than in healthy controls. Line in the scatter plots means the median. IBS-D: Iirritable bowel syndrome with diarrhea; IOD: Integral optical density.

mRNA levels were significantly increased in IBS-D patients compared to the control group. Complex factors influencing serum leptin levels and the relatively small sample size may have contributed to the lack of a difference in serum leptin levels.

The potential role of immune activation in the pathogenesis and symptom formation in IBS patients has been reported in previous studies[14,48,49]. Mast cells are a key component in the induction and maintenance of low-grade immune activation in IBS patients[48]. A recent report has demonstrated that leptin, a regulatory hormone that has multiple modulatory effects on immune cells, including T cells, macrophages, mast cells, dendritic cells, neutrophils, eosinophils, basophils, and NK cells, was able to promote the generation of an inflammatory phenotype of mast cells through the induction of interferon-γ and the suppression of interleukin-4[50]. In animal experiments, leptin has been proven to be effective in activating the colonic submucosal and myenteric neurons of guinea pigs[16] and in modulating the activity of enteric inhibitory and excitatory neurons in the proximal colon of rats[17]. It has also been reported that leptin can stimulate the activity of sensory afferent neurons[18]. In this study, the expression and location of leptin, leptin receptors, tryptase, and PGP9.5 were analyzed by immunohistochemistry and immunofluorescence. We found that the expression of leptin, rather than the leptin receptor, increased significantly. In addition, the colocalization of leptin and leptin receptors was observed on mast cells and PGP9.5-positive nerve fibers in the intestinal mucosa. These results indicate that leptin may be involved in the pathogenesis of IBS-D *via* mechanisms similar to those noted in the

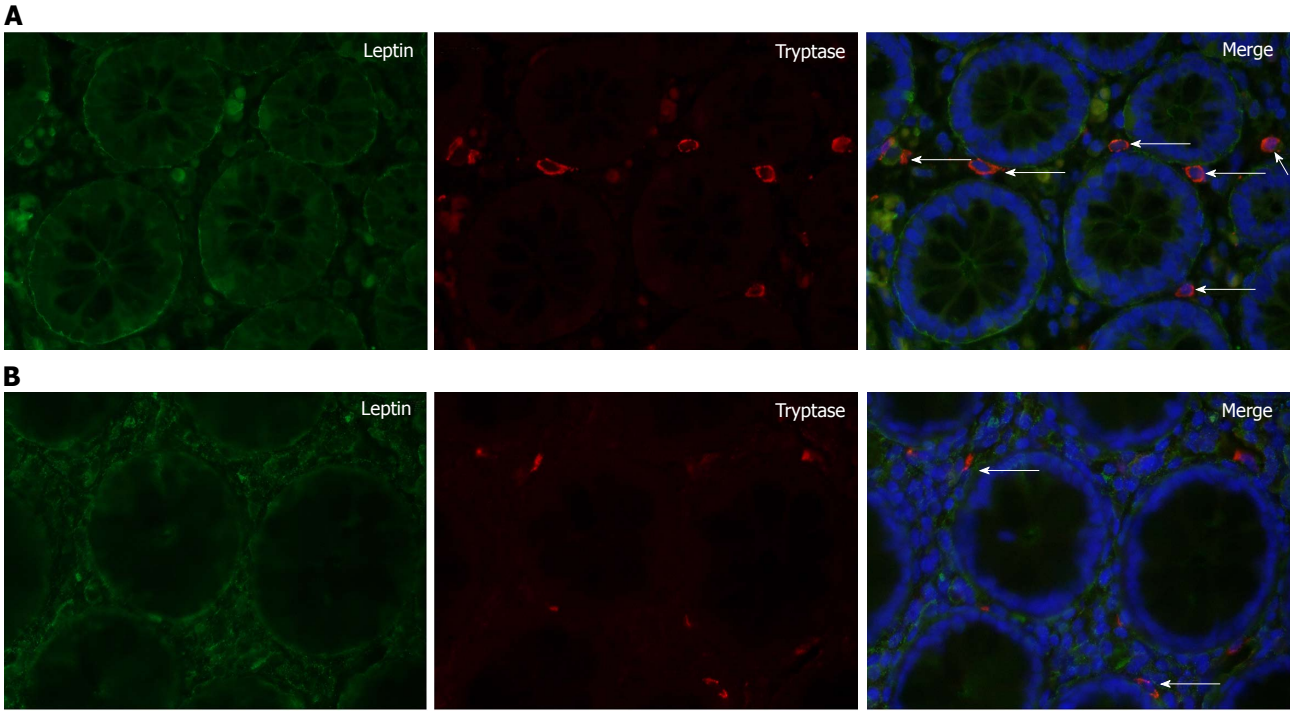

Figure 3 Double-labeling immunofluorescence of leptin and tryptase in irritable bowel syndrome patients and healthy controls. A: Colonic mucosal double-labeling immunofluorescence of leptin and tryptase in IBS-D patients (× 400, arrows represent colocalization of leptin and tryptase); B: Colonic mucosal double-labeling immunofluorescence of leptin and tryptase in healthy controls (× 400, arrows represent colocalization of leptin and tryptase). IBS-D: Iirritable bowel syndrome with diarrhea.

Table 2 Correlation between leptin level and clinical and experimental parameters in irritable bowel syndrome patients

	Leptin expression	Leptin mRNA
Questionnaire		
IBS-SSS	0.417 (0.006)[1]	0.678 (< 0.001)
HAMA	0.540 (< 0.001)	0.675 (< 0.001)
HAMD	0.488 (0.001)	0.589 (< 0.001)
Visceral sensitivity		
First sensation	-0.438 (0.004)[1]	-0.604 (< 0.001)
Defecating sensation	-0.654 (< 0.001)	-0.480 (0.001)
Maximum tolerance	-0.576 (< 0.001)	-0.706 (< 0.001)
Mucosal parameter		
Mast cell activation rate (%)	0.510 (0.001)	0.430 (0.005)[1]
Leptin mRNA	0.666 (< 0.001)	NA
Leptin expression	NA	0.666 (< 0.001)

[1]Without significance after Bonferroni correction (adjusted $P < 0.0038$). Spearman's correlation coefficients are expressed with P values in parentheses. IBS-D: Iirritable bowel syndrome with diarrhea; IBS-SSS: IBS Symptom Severity Scale; HAMA: Hamilton Anxiety Scale; HAMD: Hamilton Depression Scale; NA: Not applicable.

above studies.

Correlation analyses were performed between intestinal mucosa leptin levels and other parameters. Leptin expression was positively correlated with anxiety, depression, and the mast cell activation rate, and was negatively correlated with the defecation sensation threshold and the maximum tolerable threshold during visceral sensitivity testing. Leptin mRNA levels were positively correlated with disease severity, anxiety, and depression, and negatively correlated with the visceral sensation threshold. The correlation analyses indicate that leptin might be an important factor involved in the pathogenesis and symptom formation of IBS-D patients. However, correlations cannot be interpreted as causal associations, and further studies are needed to confirm this finding.

Based on the results of our study, we speculate that psychosocial and other stimulations cause disruption in the brain-gut axis[51]. Corticotrophin-releasing hormone (CRH) and cortisol are released with activation of the hypothalamic-pituitary axis[51]. CRH and cortisol are also increased with the production of leptin[51,52]. Also, intestinal mucosal mast cells produce histamine, serotonin, and bradykinin when activated. As a key component of low-grade mucosal inflammation, mast cells also cause increased epithelial permeability, visceral hypersensitivity, and a disruption of gastrointestinal secretory and motility functions. Leptin-stimulated mast cells modulate low-grade mucosal inflammation, activate nervous system, and increase epithelial tight junction permeability[53], all of which contribute to the development of IBS-D symptoms.

There were several limitations in this study. First, because of the nature of our research foundation[32,54], the present study focused on patients with the IBS-D subtype. Therefore, the findings may not be generalizable to constipation-predominant IBS, mixed-type IBS, and unsubtyped IBS patients. Second, leptin has been shown to increase during the process of inflammation[55]. However, we cannot be sure that all postinfective IBS patients were excluded from the

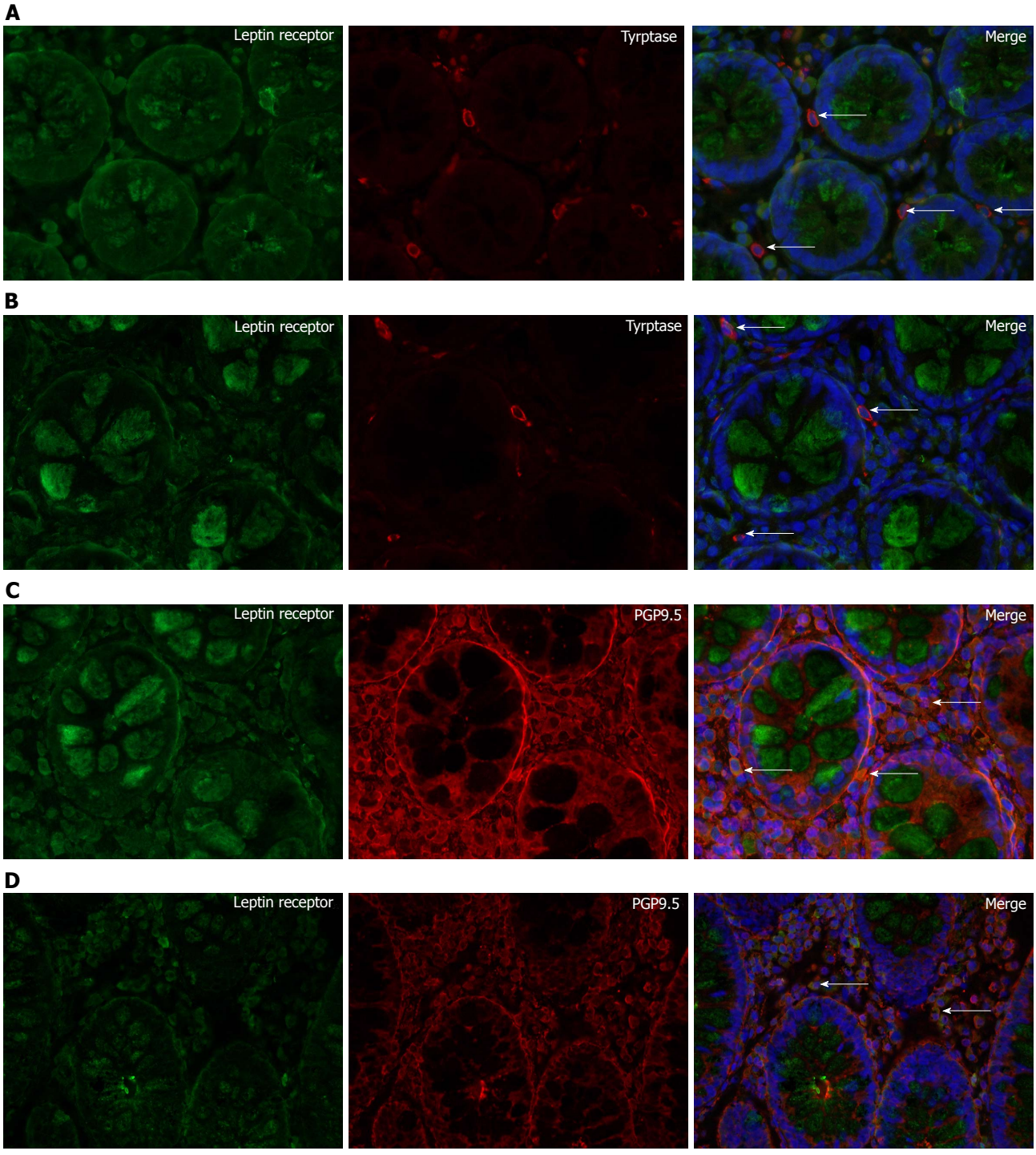

Figure 4 Double-labeling immunofluorescence of leptin receptor with tryptase and PGP9.5 in irritable bowel syndrome patients and healthy controls (× 400 magnification). A: Colonic mucosal double-labeling immunofluorescence of leptin receptor and tryptase in IBS-D patients (× 400, arrows represent colocalization of leptin receptor and tryptase); B: Colonic mucosal double-labeling immunofluorescence of leptin receptor and tryptase in healthy controls (× 400, arrows represent colocalization of leptin receptor and tryptase); C: Colonic mucosal double-labeling immunofluorescence of leptin receptor and PGP9.5 in IBS-D patients (× 400, arrows represent colocalization of leptin receptor and PGP9.5); D: Colonic mucosal double-labeling immunofluorescence of leptin receptor and PGP9.5 in healthy controls (× 400, arrows represent colocalization of leptin receptor and PGP9.5). PGP9.5: Protein gene product 9.5; IBS-D: Iirritable bowel syndrome with diarrhea.

study. Because these two subgroups may be different in pathogenesis, future experiments should study these two types separately. Third, due to the lack of previous data related to our study, we did not estimate sample size and power, which may reduce the reliability of our conclusion. Fourth, in qRT-PCR, we used only one reference gene (GAPDH), which may influence the accuracy of the results. Finally, we cannot make a cause and effect inference on the basis of our findings; additional studies of the effects of leptin on mast cells and the nervous system as well as larger clinical studies are needed.

ARTICLE HIGHLIGHTS

Research background

Irritable bowel syndrome (IBS) is a commonly diagnosed functional gastrointestinal disease. Symptoms may worsen over time and significantly impact patient quality of life and work productivity. A major subtype of IBS is IBS with diarrhea (IBS-D). The pathogenesis of IBS-D is complex and poorly understood. Leptin not only exerts significant biological effects, such as appetite control, by signaling satiety and increasing energy expenditure, but also modulates the immune system and gastrointestinal function. Few studies have specifically addressed the role of leptin in the pathogenesis of IBS. To our knowledge, this was the first study to preliminarily investigate the possible role of leptin in the pathophysiology of IBS-D.

Research motivation

The main topics of the present study include evaluating clinical symptoms, psychological characteristics, and visceral sensitivity of IBS-D patients and healthy controls; examining leptin expression, mast cells, and PGP9.5 nerve fibres in IBS-D and healthy controls; and performing correlation analyses between these parameters. Our study proposed a mechanism by which leptin may contribute to the pathophysiology of IBS.

Research objectives

This study aimed to measure leptin expression in both the serum and intestinal mucosa of patients with IBS-D subtype disease and to analyze the relationship of leptin with the clinical features, visceral sensitivity, and number of mast cells and nerve fibers in these patients.

Research methods

Participants underwent clinical and psychological evaluations using validated questionnaires (including IBS Symptom Severity Scale, IBS-specific Quality of Life, Hamilton Anxiety Scale and Hamilton Depression Scale), along with colonoscopy, colonic mucosal biopsy, and visceral sensitivity testing. Serum leptin levels were assayed using enzyme-linked immunosorbent assay. Mucosal leptin expression and localization were evaluated using immunohistochemistry and immunofluorescence. Mucosal leptin mRNA levels were quantified using quantitative real-time reverse transcription polymerase chain reaction. Mast cell counts and activation rates were investigated by toluidine blue staining. Correlation analyses between these parameters were performed. All statistical analyses were performed using SPSS for Windows software, version 24.0 (SPSS Inc, Chicago, IL).

Research results

The authors found that IBS-D patients had significantly increased psychological symptoms and visceral hypersensitivity, and their mucosal leptin expression, leptin mRNA levels, and mast cell activation rates were significantly increased. Also, leptin expression was positively correlated with anxiety, depression, and the mast cell activation rate, but negatively correlated with the defecation sensation threshold and the maximum tolerance threshold during visceral sensitivity testing. Increased levels of mucosal leptin may interact with mast cells and the nervous system to contribute to the pathogenesis of IBS-D.

Research conclusions

This study presents evidence that leptin levels are increased in the intestinal mucosa of IBS-D patients, proposes a mechanism by which leptin may contribute to the pathophysiology of IBS, and provides some potential avenues for more specific and effective treatments in these patients. The authors believe that this study makes a significant contribution to the literature because it is an original research that provides information that not only contributes to the understanding of the pathogenesis and pathophysiology of IBS, but also suggests area for future research and therapeutic intervention.

Research perspectives

This study preliminarily investigated the possible role of leptin involved in the pathogenesis of IBS-D. Future studies should focus on the following aspects. First, the present study focused on patients with the IBS-D subtype. Therefore, the findings may not be generalizable to constipation-predominant IBS, mixed-type IBS, and unsubtyped IBS patients. Future research should investigate the possible role of leptin in the pathophysiology of the other three subtypes. Second, leptin has been shown to increase during the process of inflammation. However, we cannot be sure that all postinfective IBS patients were excluded from the study. Because these two subgroups may be different in pathogenesis, future experiments should study these two types separately. Finally, we cannot make a cause and effect inference on the basis of our findings, therefore, additional studies of the effects of leptin on mast cells and the nervous system as well as larger clinical studies are needed.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Correlation between smoking habit and surgical outcomes on viral-associated hepatocellular carcinomas

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Abstract

AIM

To investigate the association between smoking habits and surgical outcomes in hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC) (B-HCC) and hepatitis C virus (HCV)-related HCC (C-HCC) and clarify the clinicopathological features associated with smoking status in B-HCC and C-HCC patients.

METHODS

We retrospectively examined the cases of the 341 consecutive patients with viral-associated HCC (C-HCC, *n* = 273; B-HCC, *n* = 68) who underwent curative surgery for their primary lesion. We categorized smoking status at the time of surgery into never, ex- and current smoker. We analyzed the B-HCC and C-HCC groups' clinicopathological features and surgical outcomes, *i.e.*, disease-free survival (DFS), overall survival (OS), and disease-specific survival (DSS). Univariate and multivariate analyses were performed using a Cox proportional hazards regression model. We also performed subset analyses in both patient groups comparing the current smokers to the other patients.

RESULTS

The multivariate analysis in the C-HCC group revealed that current-smoker status was significantly correlated with both OS (*P* = 0.0039) and DSS (*P* = 0.0416). In the B-HCC patients, no significant correlation was observed between current-smoker status and DFS, OS, or DSS in the univariate or multivariate analyses. The subset analyses comparing the current smokers to the other patients in both the C-HCC and B-HCC groups revealed that the current smokers developed HCC at significantly younger ages than the other patients irrespective of viral infection status.

CONCLUSION

A smoking habit is significantly correlated with the overall and disease-specific survivals of patients with C-HCC. In contrast, the B-HCC patients showed a weak association between smoking status and surgical outcomes.

Key words: Hepatocellular carcinoma; Hepatitis B virus; Hepatitis C virus; Smoking; Surgery; Prognosis

Core tip: We retrospectively analyzed the association between smoking habits and surgical outcomes in 68 cases of hepatitis B virus-related hepatocellular carcinoma (HCC) (B-HCC) and 273 cases of hepatitis C virus (HCV)-related HCC (C-HCC). Smoking habit was revealed as significantly correlated with the overall survival and disease-specific survival of the C-HCC patients, whereas the B-HCC patient group showed a weak association between smoking habit and surgical outcomes. Our subset analyses comparing the current smokers to the other patients revealed that the current smokers developed HCC at significantly younger ages compared to the other patients irrespective of viral infection status.

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INTRODUCTION

Hepatocellular carcinoma (HCC), is the fifth most common cancer in the world and the second most common cause of cancer deaths, accounting for about 745000 deaths per year globally[1]. Chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections represent the leading cause of HCC (60%-70%), with a total incidence of 16/100000 globally[2]. The prevalence of HBV and HCV infections varies among geographic regions. In Japan, HCV-related HCC (C-HCC) is the most common HCC infection, accounting for approx. 70% of all HCC cases[3].

It is reported that the natural histories of C-HCC and HBV-related HCC (B-HCC) differ: HCV infection leads to the development of HCC mainly through indirect pathways (such as chronic inflammation, cell deaths and proliferation) whereas HBV causes HCC through direct and indirect pathways as it can integrate into the host genome, affecting cellular signaling and growth control[4]. HCC is thus typically found in HCV patients with cirrhosis, although it is sometimes found in HBV patients without significant liver cirrhosis.

Curative surgical resection is the most frequent therapeutic strategy for HCC. The influence of the patients' viral infection status on surgical outcomes has been well investigated. A meta-analysis in 2011 reported that non-B non-C (NBNC)-HCC patients showed significantly better disease-free survival (DFS) surgical outcomes; in addition, although the result was not statistically significant, the overall survival (OS) of NBNC-HCC patients was more favorable compared to that of both B-HCC and C-HCC patients[5]. This meta-analysis of 20 studies also reported that the OS and the DFS were not significantly different between B-HCC and C-HCC groups[5]. A Japanese nationwide study of nearly 12000 patients also reported that both the OS and DFS after surgical resection were not significantly different between the C-HCC and B-HCC groups, although the liver function in the C-HCC group was significantly worse than that in the B-HCC group[6].

Generally, lifestyle-associated factors such as metabolic disease, alcohol consumption, and smoking status have tended to be left out of investigations when discussing factors affecting the pathogenesis or surgical outcomes of viral-associated HCC. It is well known that lifestyle factors are significantly involved in the carcinogenesis of NBNC-HCC and it has been reported that metabolic factors correlate also with prognosis of NBNC-HCC[7]. Although epidemiological studies obtained evidence that smoking habit is involved in HCC carcinogenesis[8-10], little attention has

been paid to the relationship between smoking habit and surgical outcomes of HCC.

We recently analyzed the relationship between smoking status and surgical outcomes in patients with NBNC-HCC, and our analysis revealed that smoking habits are significantly correlated with the curatively resected surgical outcomes of NBNC-HCC[11]. We then speculated that if smoking habits truly affect the postoperative prognosis of NBNC-HCC by one or more unknown mechanisms, smoking habits might also affect the postoperative prognosis of viral-associated HCC patients. In addition, since the natural histories of NBNC-HCC, B-HCC, and C-HCC differ, the clinicopathologic characteristics associated with smoking status might be different per viral infection status.

We thus conducted the present study to (1) investigate the association between smoking habits and surgical outcomes in B-HCC and C-HCC patients who underwent curative surgery; and (2) clarify the clinicopathological features associated with smoking habits in patients with B-HCC or C-HCC.

MATERIALS AND METHODS

The patient series and our definition of smoking status

This retrospective study's protocol was reviewed by the Ethics Committee of the Faculty of Medicine at Saga University and approved (approval No. 28-23). The written informed consent for the use of their clinical information was obtained from all of the study's patients. From 1984 to 2012, consecutive 477 cases of curative surgery for primary HCC at Saga University Hospital (in the city of Saga, which is located on the island of Kyushu, the southwestern-most of Japan's main islands) were initially enrolled the study. Definition of the HBV infection and HCV infection was HBsAg-positive and HCVAb-positive in serological tests, respectively. We excluded the following patients: those with NBNC-HCC (serologically both HBsAg- and HCVAb-negative) cases (n = 83) and those with co-infection of HBV with HCV (n = 9). Among the remaining 385 cases of C-HCC or B-HCC, we included only the cases for which all of the following information was available: the patient's age, gender, body mass index (BMI), diabetes mellitus status, smoking status (as defined below), alcohol abuse status, tumor size, status of portal vein invasion (Vp), number of primary tumors (solitary or multiple), T factor of the TMN classification, indocyanine green retention rate at 15 minutes (ICG R15), and serum alpha-fetoprotein (AFP) level. The final patient series was comprised of the 341 patients with viral-associated HCC (C-HCC, n = 273; B-HCC, n = 68).

We obtained the information about smoking status and alcohol abuse status from the patients' medical records. This information had been self-reported by the patients in an interview by medical staff. Each patient's smoking status at the time of surgery was categorized into never smoker, ex-smoker, and current smoker based on the definitions in our recent study[11], as follows. 'Never smoker' is self-explanatory. 'Ex-smoker' was defined as having quit smoking completely $\geqslant$ 1 year before the patient's surgery. Definition of 'Current smoker' was an individual who continued to smoke within 1 year prior to the surgery.

We have defined alcohol abuse as a daily ethanol intake > 40 g for men and > 20 g for women.

Statistical analyses

Statistical analyses were performed by the authors Komukai S and Kawaguchi A, who are statisticians. The software JMP ver. 12.2 and SAS ver. 9.4 (SAS, Cary, NC, United States) were used for the statistical analyses. They compared pairs of groups by Fisher's exact test, the χ^2 test and Student's t-test, as appropriate. The patients' DFS, OS and disease-specific survival (DSS) were determined as described[11]. A univariate analysis and a multivariate analysis were performed using a Cox proportional hazards regression model.

The multivariate analysis was conducted in order to adjust the potential covariates in the comparison of smoking status groups; the patients' age and gender were always kept in the model, and other parameters were identified by the stepwise procedure using the P value threshold of 0.2. The complete patient series' median age (67 years old) was used as the age cut-off. The Kaplan-Meier method was used for calculating each of the postoperative survival curves. The log-rank test was used to compare the differences in survival curves. P value < 0.05 were accepted as significant.

RESULTS

Comparison of clinicopathological features and surgical outcomes between the HBV-related HCC and HCV-related HCC patients

The clinicopathological features of 273 cases of C-HCC and 68 cases of B-HCC are summarized in Table 1. The B-HCC group developed HCC at significantly younger ages (mean 57.15 years old) compared to the C-HCC group (mean: 67.16 years, P < 0.0001). Both the C- and B-HCC groups showed a male predominance, and the B-HCC patients showed a higher male predominance rate (86.76%) compared to the C-HCC patients (74.36%, P = 0.03).

Regarding smoking status, no significant difference was observed between the two groups although the B-HCC group tended to have more current smokers (47.06%) compared to the C-HCC group (32.23%). The status of alcohol abuse, diabetes mellitus, and BMI did not differ between the C- and B-HCC patients.

The percentage of ICG R15 was significantly higher in the C-HCC group compared to the B-HCC group (P < 0.0001), indicating that the patients with HCV infection developed HCC at a more advanced stage of chronic hepatitis compared to the patients with HBV infection. The serum AFP level of at the time of surgery tended to be higher in the B-HCC patients compared to the

Table 1 Clinicopathologic features of the hepatitis C virus-hepatocellular carcinoma and hepatitis B virus-hepatocellular carcinoma *n* (%)

	HCV (*n* = 273)	HBV (*n* = 68)	*P* value
Age (mean ± SD)	67.16 ± 8.56	57.15 ± 12.47	< 0.0001
Gender			
Male	203 (74.36)	59 (86.76)	0.0300
Female	70 (25.64)	9 (13.24)	
Smoking habit			
Never	111 (40.66)	21 (30.88)	0.0715
Ex	74 (27.11)	15 (22.06)	
Current	88 (32.23)	32 (47.06)	
Alcohol abuse			
(+)	63 (23.08)	22 (32.35)	0.1136
(-)	210 (76.92)	46 (67.65)	
Diabetes mellitus			
(+)	62 (22.71)	12 (17.65)	0.3647
(-)	211 (77.29)	56 (82.35)	
BMI (mean ± SD)	22.49 ± 3.29	22.93 ± 3.80	0.3478
ICG R15 (%) (mean ± SD)	18.63 ± 9.03	13.31 ± 6.30	< 0.0001
AFP (ng/mL), Median (range)	26.2 (0, 29800)	31 (1, 271600)	0.0625
Tumor size (mean ± SD mm)	37.37 ± 25.62	49.72 ± 33.14	0.0052
Solitary/Multiple			
Solitary	182 (66.67)	41 (60.29)	0.3230
Multiple	91 (33.33)	27 (39.71)	
Vp			
(+)	74 (27.11)	29 (42.65)	0.0125
(-)	199 (72.89)	39 (57.35)	
T factor			
T1/2	156 (57.14)	32 (47.06)	0.1347
T3/4	117 (42.86)	36 (52.94)	

HCV: Hepatitis C virus; HBV: Hepatitis B virus.

C-HCC patients, but the difference was not significant. The tumor sizes in the B-HCC group were significantly larger than those of the C-HCC group (mean tumor sizes 49.72 mm *vs* 37.37 mm, $P = 0.0052$), and the percentage of Vp was significantly higher in the B-HCC group compared to the C-HCC (42.65% *vs* 27.11%, $P = 0.0125$). There was no significant difference regarding T factor or multiple occurrence between the B-and C-HCC groups.

We compared the surgical outcomes (DFS, OS, DSS) of the B-HCC and C-HCC groups and found no significant difference between the groups in DFS, OS or DSS (Figure 1).

Univariate analysis results and survival curves per smoking status in the HCV-related HCC patients

The results of the univariate analyses for surgical outcomes in the C-HCC patient group are summarized in Table 2. The factors that were significantly correlated with the DFS of the C-HCC patient group were alcohol abuse ($P = 0.0321$), BMI ($P = 0.0270$), ICG R15 ($P = 0.0088$), tumor size ($P = 0.0305$), multiple tumors ($P = 0.0021$), and T factor ($P < 0.0001$). Smoking status was not correlated with the DFS of the C-HCC patients.

The factors that were revealed to be significantly correlated with the OS of the C-HCC patient group were smoking (current *vs* other; $P = 0.0144$), serum AFP level ($P = 0.0402$), tumor size ($P = 0.0019$), multiple tumors ($P = 0.0001$), Vp ($P = 0.0004$), and T factor ($P = 0.0008$).

The factors found to be significantly correlated with disease-specific survival were smoking (current *vs* other; $P = 0.0483$), tumor size ($P = 0.0070$), Vp ($P = 0.0177$), and T factor ($P = 0.0315$). The survival curves per smoking habit are demonstrated in Figure 2A-C. The current-smoking group showed significantly poor survival curves compared to the never + Ex patient group for both OS and DSS. However, no significant difference was observed in DFS between the current-smoking group and never + Ex patient group.

Multivariate analysis results per smoking status in HCV-related HCC patients

The results of the multivariate analyses for DFS, OS and DSS are summarized in Table 3. Current-smoker status showed no correlation with DFS ($P = 0.2364$). The factors that were significantly correlated with DFS were alcohol abuse ($P = 0.0025$), BMI ($P = 0.0165$), ICG R15 ($P = 0.0027$) and T factor ($P < 0.0001$). In the multivariate analysis for OS, current-smoker status was significantly correlated with OS ($P = 0.0039$). The only other factor that was significantly correlated with OS was T factor ($P = 0.0005$). The factors significantly correlated with DSS were current-smoker status and T factor ($P = 0.0416$ and $P = 0.0226$, respectively).

Univariate analysis results and survival curves for the HBV-related HCC patients per smoker status

The results of the univariate analyses for surgical outcomes in the B-HCC group are summarized in Table

Table 2 Univariate analysis results: Disease-free, overall and disease-specific survival after hepatic resection for hepatocellular carcinoma (hepatitis C virus, *n* = 273)

		DFS		OS		DSS	
Characteristics	***n***	**HR (95%CI)**	***P* value**	**HR (95%CI)**	***P* value**	**HR (95%CI)**	***P* value**
Age			0.9234		0.5143		0.7101
⩽ 67	133	1		1		1	
> 67	140	1.01381 (0.7665, 1.3409)		1.10679 (0.8159, 1.5015)		0.92155 (0.599, 1.4177)	
Gender			0.5699		0.5110		0.2434
Female	70	1		1		1	
Male	203	0.91269 (0.666, 1.2508)		1.12916 (0.786, 1.6221)		1.38366 (0.8019, 2.3876)	
Smoking habit (Ex + current)			0.5978		0.1262		0.2147
Absent	111	1		1		1	
Present	162	0.92696 (0.6994, 1.2286)		1.27849 (0.9332, 1.7516)		1.32797 (0.8483, 2.0788)	
Smoking habit (current)			0.8864		0.0144		0.0483
Absent	185	1		1		1	
Present	88	1.02258 (0.7527, 1.3892)		1.47783 (1.0808, 2.0207)		1.55795 (1.0033, 2.4192)	
Alcohol abuse			0.0321		0.8232		0.7027
Absent	210	1		1		1	
Present	63	0.68132 (0.4797, 0.9678)		0.95952 (0.6677, 1.3788)		0.90325 (0.5356, 1.5232)	
Diabetes mellitus			0.3149		0.5259		0.9487
Absent	211	1		1		1	
Present	62	1.18657 (0.85, 1.6564)		1.12207 (0.7861, 1.6017)		0.98302 (0.5831, 1.6572)	
BMI			0.0270		0.2082		0.6409
⩽ Median	137	1		1		1	
> Median	136	0.72863 (0.5503, 0.9647)		0.82193 (0.6056, 1.1155)		0.9026 (0.5868, 1.3884)	
ICG R15 (%)			0.0088		0.2391		0.6896
⩽ Median	111	1		1		1	
> Median	162	1.47199 (1.1021, 1.9659)		1.20672 (0.8825, 1.65)		1.09334 (0.7056, 1.6941)	
AFP			0.2274		0.0402		0.1371
⩽ Median	136	1		1		1	
> Median	137	1.18795 (0.8981, 1.5713)		1.37689 (1.0144, 1.8689)		1.38776 (0.901, 2.1376)	
Tumor size			0.0305		0.0019		0.0070
⩽ Median	161	1		1		1	
> Median	112	1.37235 (1.0303, 1.828)		1.63014 (1.1982, 2.2178)		1.81608 (1.1773, 2.8016)	
Solitary/Multiple			0.0021		0.0001		0.1182
Solitary	182	1		1		1	
Multiple	91	1.59365 (1.1835, 2.1459)		1.8536 (1.3563, 2.5332)		1.43932 (0.9115, 2.2728)	
Vp			0.0767		0.0004		0.0177
Absent	199	1		1		1	
Present	74	1.33528 (0.9695, 1.839)		1.81728 (1.3073, 2.5262)		1.76737 (1.104, 2.8294)	
T12/T34			< 0.0001		0.0008		0.0315
T12	156	1		1		1	
T34	117	1.81021 (1.3644, 2.4017)		1.68381 (1.2412, 2.2843)		1.60589 (1.0429, 2.4728)	

DFS: Disease-free survival; OS: Overall survival; DSS: Disease-specific survival.

4. The factors that were significantly correlated with the DFS of the B-HCC patient group were alcohol abuse (P = 0.0183), multiple tumors (P = 0.0002) and T factor (P = 0.0042). The factors significantly correlated with the OS of the B-HCC group were the serum AFP level (P = 0.0015), multiple tumors (P = 0.0001), Vp (P = 0.0035), and T factor (P = 0.0001). The factors revealed to be significantly correlated with disease-specific survival were the serum AFP level (P = 0.0114), multiple tumors (P = 0.0013), Vp (P = 0.0362), and T factor (P = 0.0019).

The survival curves of DFS, OS and DSS per smoking status are demonstrated in Figure 2D-F. Smoking status was not correlated with the DFS, OS or DSS of the B-HCC patients.

Multivariate analysis results regarding smoking status in the HBV-related HCC patients

The results of the multivariate analyses are summarized in Table 5. The two factors that were significantly correlated with the DFS of the B-HCC patients were alcohol abuse (P = 0.0119) and multiple tumors (P = 0.0004). The factors that were significantly correlated with the patients' OS were alcohol abuse (P = 0.0312) and multiple tumors (P = 0.0001). The only factor that was significantly correlated with disease-specific survival was multiple tumors (P = 0.0009). Current-smoker status showed no correlation with any DFS, OS or DSS.

Comparison of clinicopathological factors per current-smoker status in the HCV-related HCC and HBV-related HCC groups

To clarify the clinicopathological characteristics of the current smokers in the C-HCC and B-HCC groups, we performed subset analyses regarding the clinicopathological factors per current-smoker status (Table 6).

Among the C-HCC patients, the current smokers

Table 3 Multivariate analyses for current smokers *vs* others (hepatitis C virus, *n* = 273)

Type	Characteristics	HR (95%CI)	*P* value
DFS	Smoking habit (current)	1.22892 (0.8736, 1.7287)	0.2364
	Age (67 < yr)	0.98815 (0.7442, 1.3121)	0.9343
	Gender (male)	0.9536 (0.6797, 1.338)	0.7833
	Alcohol abuse	0.55622 (0.3803, 0.8135)	0.0025
	BMI (median <)	0.70403 (0.5284, 0.9381)	0.0165
	ICG R15 (median <)	1.59531 (1.1763, 2.1635)	0.0027
	T factor (T3/4)	1.90638 (1.4279, 2.5452)	< 0.0001
OS	Smoking habit (current)	1.69259 (1.1844, 2.4187)	0.0039
	Age (67 < yr)	1.18434 (0.8668, 1.6182)	0.2880
	Gender (male)	1.01654 (0.687, 1.504)	0.9346
	Alcohol abuse	0.74612 (0.5061, 1.1)	0.1392
	BMI (median <)	0.89005 (0.6489, 1.2208)	0.4701
	ICG R15 (median <)	1.32015 (0.9524, 1.83)	0.0955
	T factor (T3/4)	1.74555 (1.2767, 2.3865)	0.0005
DSS	Smoking habit (current)	1.68394 (1.0201, 2.7798)	0.0416
	Age (67 < yr)	1.00555 (0.6464, 1.5644)	0.9804
	Gender (male)	1.25081 (0.6981, 2.2412)	0.4520
	Alcohol abuse	0.66203 (0.3796, 1.1547)	0.1462
	BMI (median <)	0.97081 (0.6206, 1.5185)	0.8967
	ICG R15 (median <)	1.18278 (0.7484, 1.8692)	0.4722
	T factor (T3/4)	1.67615 (1.0752, 2.613)	0.0226

DFS: Disease-free survival; OS: Overall survival; DSS: Disease-specific survival.

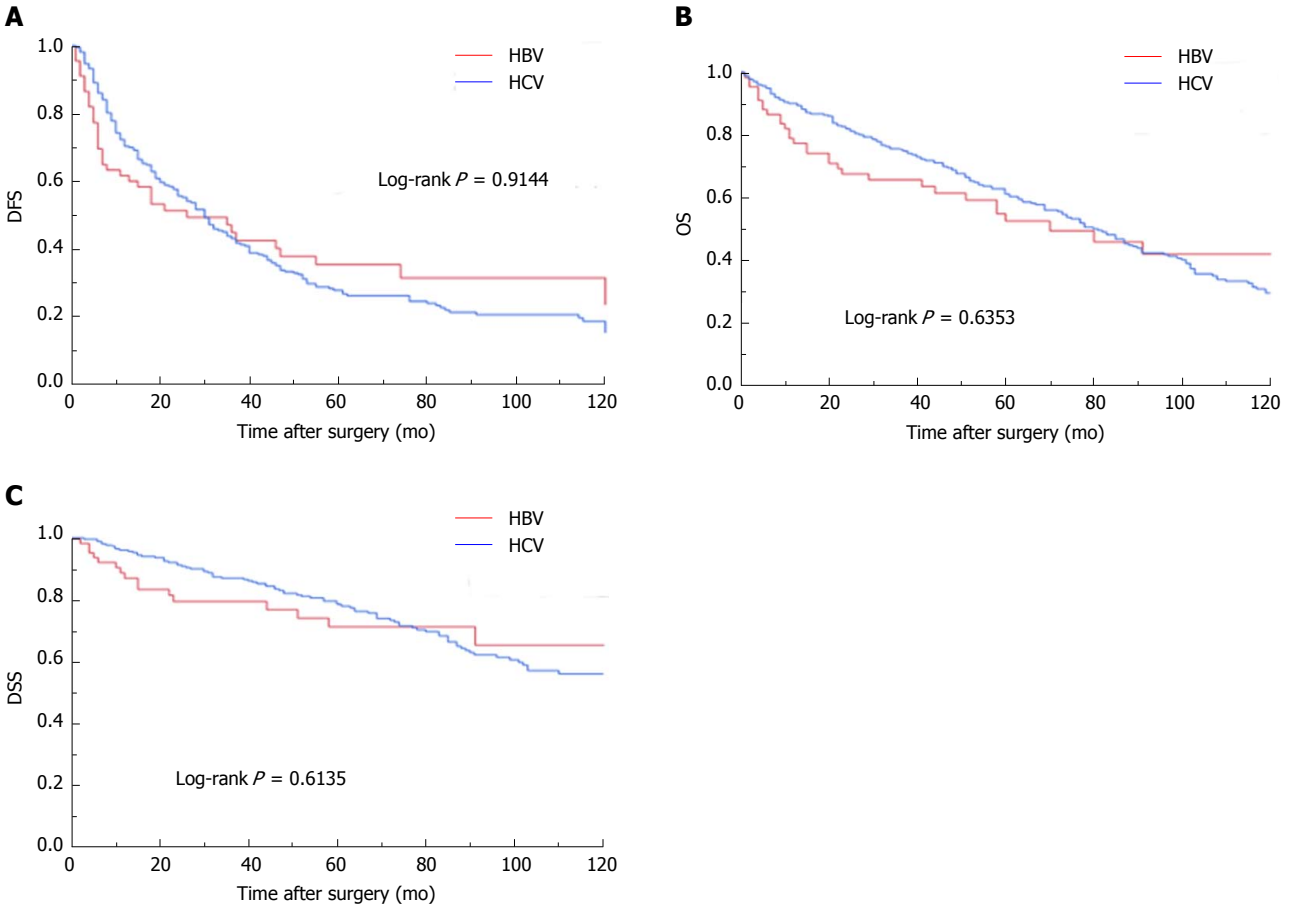

Figure 1 Kaplan-Meier curves according to viral infection status for disease-free survival (A), overall survival (B) and disease-specific survival (C). DFS: Disease-free survival; OS: Overall survival; DSS: Disease-specific survival.

were slightly but significantly younger than the never + Ex patients (mean age 65.34 years *vs* 68.02 years, $P = 0.0153$) at the time of surgery. The current smokers showed significant male predominance ($P < 0.0001$) and had a significantly greater incidences of alcohol abuse ($P < 0.0001$). The BMI and ICG R15 values of the current smokers were both significantly lower than those of the never+Ex patients ($P = 0.0031$

Table 4 Univariate analysis results: Disease-free, overall and disease-specific survival after hepatic resection for hepatocellular carcinoma (hepatitis B virus, *n* = 68)

		DFS		OS		DSS	
Characteristics	***n***	**HR (95%CI)**	***P* value**	**HR (95%CI)**	***P* value**	**HR (95%CI)**	***P* value**
Age			0.9417		0.8991		0.3769
≤ 67	54	1		1		1	
> 67	14	0.97131 (0.445, 2.1202)		1.05615 (0.4541, 2.4565)		0.5123 (0.1162, 2.2588)	
Gender			0.6063		0.1844		0.3363
Female	9	1		1		1	
Male	59	1.28118 (0.4993, 3.2876)		2.64152 (0.6294, 11.0865)		2.70202 (0.3562, 20.4981)	
Smoking habit (Ex + current)			0.8323		0.5660		0.9544
Absent	21	1		1		1	
Present	47	0.93056 (0.478, 1.8115)		1.26612 (0.5656, 2.8343)		0.96953 (0.336, 2.7975)	
Smoking habit (current)			0.4008		0.3620		0.8001
Absent	36	1		1		1	
Present	32	1.30547 (0.7009, 2.4314)		1.38988 (0.6848, 2.8209)		1.13501 (0.4258, 3.0251)	
Alcohol abuse			0.0183		0.0706		0.5674
Absent	46	1		1		1	
Present	22	0.4139 (0.199, 0.861)		0.45823 (0.1966, 1.0679)		0.73339 (0.2534, 2.1228)	
Diabetes mellitus			0.4682		0.6700		0.8752
Absent	56	1		1		1	
Present	12	1.32065 (0.6229, 2.7999)		1.21513 (0.4959, 2.9773)		1.10618 (0.3141, 3.8962)	
BMI			0.3087		0.9126		0.6383
≤ Median	33	1		1		1	
> Median	35	1.3863 (0.7392, 2.6)		0.96125 (0.4746, 1.9468)		1.26746 (0.4718, 3.4046)	
ICG R15 (%)			0.6240		0.8562		0.5468
≤ Median	49	1		1		1	
> Median	19	0.83516 (0.4064, 1.7163)		1.07744 (0.4808, 2.4147)		1.38526 (0.4799, 3.9983)	
AFP			0.0665		0.0015		0.0114
≤ Median	34	1		1		1	
> Median	34	1.80218 (0.9606, 3.3809)		3.54578 (1.6235, 7.7443)		4.36412 (1.3942, 13.6606)	
Tumor size			0.1252		0.2124		0.0585
≤ Median	28	1		1		1	
> Median	40	1.67393 (0.8665, 3.2339)		1.61822 (0.7594, 3.4483)		3.37156 (0.9574, 11.8735)	
Solitary/Multiple			0.0002		0.0001		0.0013
Solitary	41	1		1		1	
Multiple	27	3.30417 (1.7552, 6.2201)		4.6829 (2.1913, 10.0073)		6.42975 (2.0644, 20.0257)	
Vp			0.0598		0.0035		0.0362
Absent	39	1		1		1	
Present	29	1.85208 (0.975, 3.5182)		2.94188 (1.4258, 6.07)		2.92487 (1.0717, 7.9823)	
T12/T34			0.0042		0.0001		0.0019
T12	32	1		1		1	
T34	36	2.60213 (1.3531, 5.0043)		5.55785 (2.3542, 13.1211)		10.58522 (2.3795, 47.0876)	

DFS: Disease-free survival; OS: Overall survival; DSS: Disease-specific survival.

and 0.0005, respectively). No significant difference was observed in diabetes mellitus, serum AFP level, tumor size, multiple tumor, Vp, T factor, or recurrence between the current smokers and the other patients.

In the B-HCC patient group, although the current smokers were significantly younger (mean age 53.66 years *vs* 60.25 years, $P = 0.0284$) and showed a significant male predominance ($P = 0.0204$), no significant difference was observed in any of the other factors between the current smokers and the other patients.

DISCUSSION

There have been many studies regarding cigarette smoking and the risk of developing HCC, and a recent meta-analysis confirmed the relationship between smoking and an increased risk of HCC development and mortality from HCC[12]. Including our previous study[11], there have been only a few studies focusing on the correlation between smoking status and the surgical outcomes of hepatectomy or liver transplantation for HCC[12-15]. The present study is the first to compare C-HCC and B-HCC regarding smoking status and surgical outcomes.

The most salient finding of this study is that the correlations between smoking status and surgical outcomes were notably different between the B-HCC and C-HCC patient groups. Although the current-smoking habit affected the surgical outcomes of the C-HCC patients, no significant association was found between smoking status and surgical outcomes in the B-HCC patients. As the current-smoking habit was revealed as an independent prognostic factor for both the OS and disease-specific survival in our C-HCC group, the tumors that had developed in the current smokers were indicated to have more aggressive malignant potential than the tumors that developed in the never- and ex-smokers. The continued elucidation of the

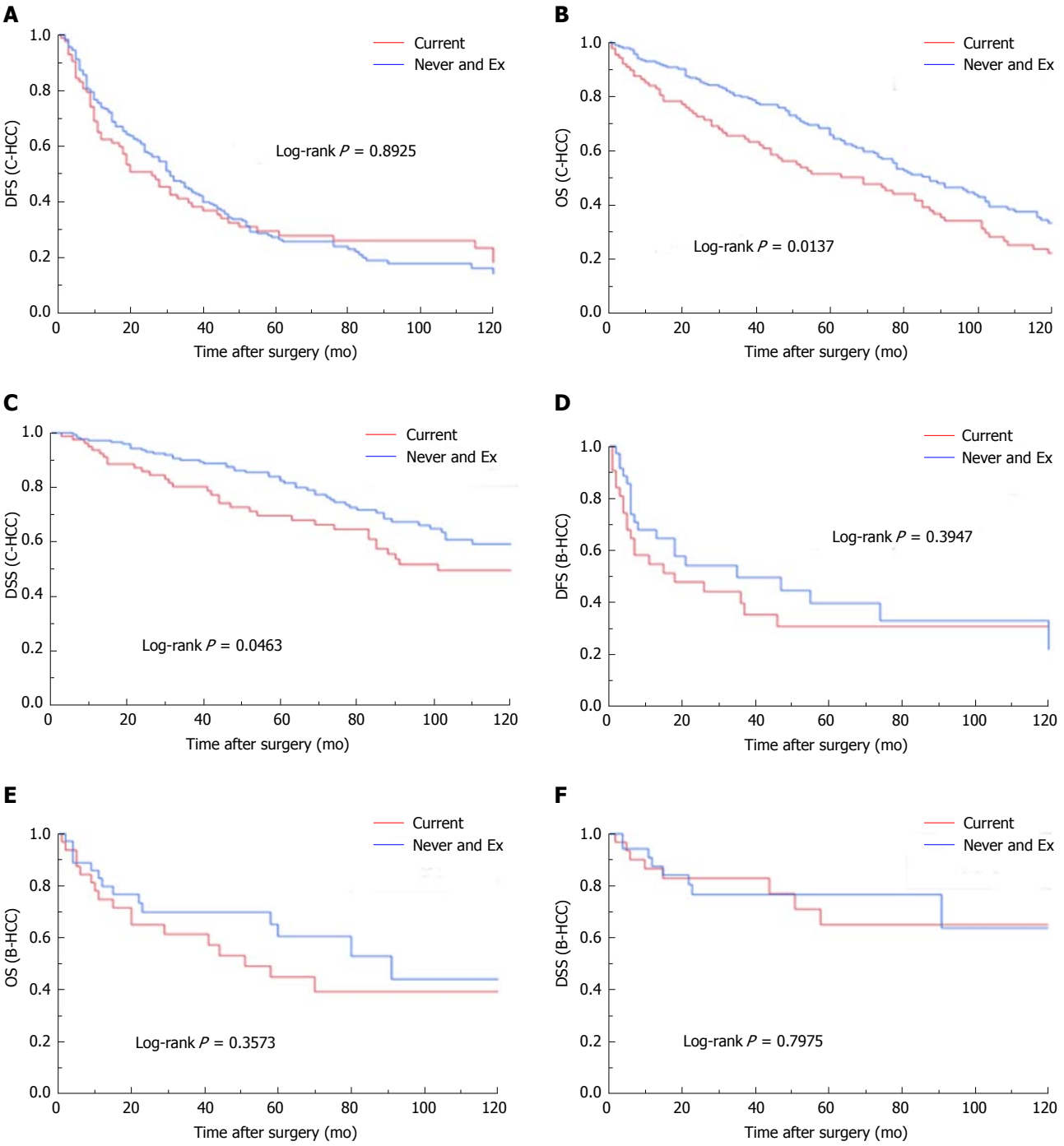

Figure 2 Kaplan-Meier curves according to smoking status for disease-free survival, overall survival and disease-specific survival in the hepatitis C virus -related hepatocellular carcinoma patients (A-C) and hepatitis B virus-related hepatocellular carcinoma patients (D-F).

pathological and molecular mechanisms underlying the development is a very important research focus, and we suspect that the difference in the natural histories of C-HCC and B-HCC is a key factor in the difference in the smoking status and surgical outcomes between B-HCC and C-HCC.

Generally, HCV-infected individuals develop HCC after long-term chronic hepatitis, and C-HCCs are typically found in patients with cirrhosis[16]. Activated inflammatory cells release reactive oxygen species and induce lipid peroxidation, which promotes a pro-oncogenic environment and DNA damage[17] and increases DNA methylation[18,19]. Thus, C-HCC develops mainly *via* an indirect pathway caused by chronic inflammation and an epigenetic process.

Although the mechanism of the carcinogenesis of HCC due to smoking has not been fully elucidated, it is reported that smoking yields chemicals with oncogenic potential such as hydrocarbons, nitrosamine, tar and vinyl chloride and a major source of 4-aminobiphenyl, a hepatic carcinogen which has been implicated as a causal risk factor for HCC[20]. These oncogenic chemicals

Table 5 Multivariate analyses for current smokers *vs* others (hepatitis B virus, *n* = 68)

Type	Characteristics	HR (95%CI)	*P* value
DFS	Smoking habit (current)	1.41417 (0.7413, 2.698)	0.2930
	Age (67 < yr)	0.9339 (0.4189, 2.0818)	0.8672
	Gender (male)	1.82154 (0.6845, 4.8477)	0.2298
	Alcohol abuse	0.37957 (0.1785, 0.8072)	0.0119
	Multiple tumor	3.18518 (1.6783, 6.045)	0.0004
OS	Smoking habit (current)	1.43613 (0.6865, 3.0045)	0.3365
	Age (67 < yr)	0.88873 (0.3727, 2.119)	0.7902
	Gender (male)	3.66949 (0.8392, 16.0457)	0.0842
	Alcohol abuse	0.38587 (0.1622, 0.9177)	0.0312
	Multiple tumor	4.91853 (2.2445, 10.7785)	0.0001
DSS	Smoking habit (current)	0.98786 (0.3539, 2.7572)	0.9814
	Age (67 < yr)	0.37251 (0.0808, 1.7181)	0.2055
	Gender (male)	3.89167 (0.4809, 31.4908)	0.2027
	Alcohol abuse	0.98786 (0.3539, 2.7572)	0.3991
	Multiple tumor	7.08829 (2.2387, 22.4436)	0.0009

DFS: Disease-free survival; OS: Overall survival; DSS: Disease-specific survival.

Table 6 Comparison of clinicopathological factors per current smoking status in the hepatitis C virus-related hepatocellular carcinoma and hepatitis B virus-related hepatocellular carcinoma patient group

	HCV (*n* = 273)			HBV (*n* = 68)		
	Current (*n* = 88)	Never + Ex (*n* = 185)	*P* value	Current (*n* = 32)	Never + Ex (*n* = 36)	*P* value
Age (mean ± SD)	65.34 ± 7.82	68.02 ± 8.77	0.0153	53.66 ± 13.74	60.25 ± 10.46	0.0284
Gender (male/female)	81/7	122/63	< 0.0001	31/1	28/8	0.0204
Alcohol abuse (+/-)	35/53	28/157	< 0.0001	13/19	9/27	0.1692
Diabetes mellitus (+/-)	20/68	42/143	0.9964	5/27	7/29	0.6801
BMI (mean ± SD)	21.71 ± 2.74	22.87 ± 3.47	0.0031	23.01 ± 3.89	22.85 ± 3.77	0.8656
ICG R15 (%)	16.14 ± 7.24	19.81 ± 9.56	0.0005	12.54 ± 5.25	14.00 ± 7.11	0.3451
AFP (ng/mL), median (range)	20.5 (2, 29800)	29 (0, 19500)	0.4338	64.9 (1, 271600)	14.8 (2.4, 209900)	0.7995
Tumor size (mean ± SD mm)	39.25 ± 25.87	36.48 ± 25.52	0.4050	47.69 ± 29.26	51.53 ± 36.56	0.6369
Solitary/Multiple	60/28	122/63	0.7142	17/15	24/12	0.2546
Vp (+/-)	28/60	46/139	0.2271	13/19	16/20	0.7506
T factor (T12/T34)	48/40	108/77	0.5498	15/17	17/19	0.9772
Recurrence (+/-)	59/29	138/47	0.1934	20/12	20/16	0.5614

HCV: Hepatitis C virus; HBV: Hepatitis B virus; BMI: Body mass index; AFP: Alpha-fetoprotein.

covalently bind to DNA and form DNA adducts, which play a central role in the carcinogenic process by causing miscoding events in critical genes[21]. One hypothesis is that in current smokers, these genetic abnormalities due to smoking are superimposed on the natural course of C-HCC and thus highly malignant HCC develops. We suspect that this hypothesis will be verified by further studies in the near future.

Another interesting result of the present study is that smoking status was not correlated with DFS in the C-HCC group. Although this may be explained by the carcinogenesis *via* chronic HCV infection, it seems to contradict our finding that smoking habit was significantly associated with disease-specific survival. Two hypotheses that may explain this contradiction are as follows: (1) The malignant potential of recurrent tumors in the current smokers was higher than that in the other patients; and (2) the number of cases that had relapsed as a metastatic lesion of the resected primary tumor was larger in the current-smoker group compared to the never + Ex-smoker group. To elucidate this point clearly, it is crucial to analyze surgical outcomes based on detailed information regarding post-surgery smoking cessation. Quite regrettably, such data were not available in our database.

Although the inflammation and liver damage associated with chronic hepatitis B also introduce an accumulation of genetic and epigenetic alterations, a direct effect of HBV contributes to the development of B-HCC[2]. HBV genomes can integrate into the host genome and induce chromosomal alterations and insertional mutagenesis of cancer genes[22]. Therefore, B-HCC can develop in the absence of inflammation, which is in stark contrast to C-HCC development[23]. The reason for the different association of smoking status and surgical outcomes between the B-HCC and C-HCC groups may be caused by the differences in carcinogenesis *via* HBV infection and HCV infection. However, a study based in China that analyzed the surgical outcomes of 302 patients with B-HCC reported that smoking status was correlated with both HCC recurrence and HCC mortality[13]. Therefore, the reason why smoking status did not correlate with the surgical outcomes of B-HCC in the present study may be due simply to the small sample size of the B-HCC patients (*n* = 68, *vs* 273 C-HCC patients).

The results of our subgroup analyses of the C-HCC and B-HCC patients comparing the current-smoker patients and the other patients were also interesting. The analysis of the C-HCC group revealed that the current-smoker group developed HCC at significantly younger ages compared to the never+Ex group. The current-smoker group was similarly significantly younger in the B-HCC group. These results of the present study and our NBNC-HCC study[11] both showed that current smokers develop HCC at a younger age than other patients, which suggests an additive effect of smoking on the development of HCC irrespective of the virus infection status.

The limitations of the present study are retrospective-designed study, the small number of patients, and the long study period for enrollment. Information regarding post-surgery smoking status and treatment procedure for recurrent tumor were not available. Although we believe that our results provide important information to elucidate HCC's natural history involving the patients' lifestyle, our findings should be verified by investigations that include detailed smoking information, in large retrospective or prospective studies.

In conclusion, our present findings indicate that a current-smoking habit is significantly correlated with the overall and disease-specific survivals of patients with C-HCC. In contrast, our B-HCC patient group showed a weak association between current smoking and surgical outcomes. Our analyses also revealed that the current smokers were significantly younger than the other patients irrespective of hepatitis viral infection status.

ARTICLE HIGHLIGHTS

Research background

Although cigarette smoking has been recognized as one of the risk factors for hepatocellular carcinoma (HCC), the surgical outcomes and clinicopathological characteristics according to smoking habits of HCC patients remains unclear. We investigate the association between smoking status and surgical outcomes in hepatitis B virus-related HCC (B-HCC) and HCV-related HCC (C-HCC).

Research motivation

We recently analyzed the relationship between smoking status and surgical outcomes in patients with non-B non-C (NBNC)-HCC, and our analysis revealed that smoking habits are significantly correlated with the curatively resected surgical outcomes of NBNC-HCC. We then speculated that if smoking habits truly affect the postoperative prognosis of HCC, smoking habits might also affect the postoperative prognosis of viral-associated HCC patients.

Research objectives

We conducted the present study to investigate the association between smoking habits and surgical outcomes in B-HCC and C-HCC patients who underwent curative surgery, and clarify the clinicopathological features associated with smoking habits in patients with B-HCC or C-HCC.

Research methods

Cases of the 341 consecutive patients with viral-associated HCC (C-HCC, *n* = 273; B-HCC, *n* = 68) who underwent curative surgery for their primary lesion were retrospectively examined. We categorized smoking status at the time of surgery into never, ex- and current smoker and analyzed the clinicopathological features and surgical outcomes, *i.e.*, disease-free survival (DFS), overall survival (OS), and disease-specific survival (DSS).

Research results

The multivariate analysis in the C-HCC group revealed that current-smoker status was significantly correlated with both OS and DSS. No significant correlation was observed between current-smoker status and DFS, OS, or DSS in the B-HCC patients of the univariate or multivariate analyses.

Research conclusions

Smoking habit is significantly correlated with the overall and disease-specific survivals of patients with C-HCC, and in contrast, the B-HCC patients showed a weak association between smoking status and surgical outcomes.

Research perspectives

The results of this study support the hypothesis that smoking-associated HCC is with is high malignant potential. It would be a motivation for further research. We expect future research clarify the mechanism of carcinogenesis of HCC *via* smoking. Our results also can be expected to provide further motivation for smoking cessation.

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ORIGINAL ARTICLE

Retrospective Study

Safety and efficacy of metallic stent for unresectable distal malignant biliary obstruction in elderly patients

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Abstract

AIM

To study the safety of insertion of metallic stents in elderly patients with unresectable distal malignant biliary obstruction.

METHODS

Of 272 patients with unresectable distal malignant biliary obstruction, 184 patients under the age of 80 were classified into Group A, and 88 subjects aged 80 years or more were classified into Group B. The safety of metallic stent insertion, metal stent patency period, and the obstruction rate were examined in each group.

RESULTS

In Group B, patients had a significantly worse performance status, high blood pressure, heart disease, cerebrovascular disease, and dementia; besides the rate of patients orally administered antiplatelet drugs or anticoagulants tended to be higher ($P < 0.05$). Metallic stents were successfully inserted in all patients. The median patency period was 265.000 ± 26.779 (1-965) d; 252.000 ± 35.998 (1-618) d in Group A and 269.000 ± 47.885 (1-965) d in Group B, with no significant difference between the two groups. Metallic stent obstruction occurred in 82 of the 272 (30.15%) patients; in 53/184 (28.80%) patients in Group A and in 29/88 (32.95%) of those in Group B, showing no significant difference between the two groups. Procedural accidents due to metal stent insertion occurred in 24/272 (8.8%) patients; in 17/184 (9.2%) of patients in Group A and in 7/88 (8.0%) of those in Group B, with no significant difference between the two groups, either.

CONCLUSION

These results suggested that metallic stents can be safely inserted to treat unresectable distal malignant biliary obstruction even in elderly patients aged 80 years or more.

Key words: Elderly patients; Metallic stent; Malignant biliary obstruction

Core tip: It was suggested that metallic stent insertion for unresectable distal malignant biliary obstruction in the elderly can be conducted safely and with a high success rate, without any significant difference in the occurrence of procedural accidents when compared with the non-elderly group, even though the elderly tend to have more underlying diseases.

Sakai Y, Iwai T, Shimura K, Gon K, Koizumi K, Ijima M, Chiba K, Nakatani S, Sugiyama H, Tsuyuguchi T, Kamisawa T, Maetani I, Kida M. Safety and efficacy of metallic stent for unresectable distal malignant biliary obstruction in elderly patients. *World J Gastroenterol* 2018; 24(1): 69-75 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i1/69.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i1.69

INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP) related procedure is a minimally invasive diagnostic and therapeutic method for biliary and pancreatic diseases and plays a very important role. As for biliary drainage, endoscopic drainage by ERCP is considered to be noninvasive and the first choice of treatment[1,2]. Either plastic or metallic stents are used in endoscopic drainage for patients with unresectable malignant biliary obstruction. However, it is desirable to use a metallic stent because of its long patency period and low obstruction rate, which reduce the need for retreatment and the total treatment cost[1-4]. It is considered that metallic stents can be safely inserted in patients with unresectable malignant biliary obstruction, they are useful and the drainage effect is excellent[5-8]. Yet, to our knowledge, there are no reports that examined the safety of insertion of metallic stents in the elderly with an unresectable malignant biliary obstruction. Therefore, we studied the safety and usefulness of metallic stents for unresectable distal malignant biliary obstruction in a multicenter, collaborative, retrospective study.

MATERIALS AND METHODS

There were 272 patients diagnosed with unresectable distal malignant biliary obstruction, at the 7 institutions participating in this study, from January 2012 to July 2016. Metallic stents were inserted transpapillary using the ERCP route. In all patients, endoscopic sphincterotomy (EST) was performed previous to metal stent insertion. The metallic stent used was BONA stent (Sewoon Medical Co., Ltd. Delivery system, 8Fr., 10 mm diameter, fully covered stent). Inclusion criteria were: (1) written informed consent for the study obtained previous to the endoscopic treatment; (2) patients aged 20 years or more; (3) patients hospitalized on ERCP day who could be followed for one week after ERCP; (4) patients not subjected to stomach reconstruction with Billroth Ⅱ method or Roux-en Y anastomosis; (5) patients who could orally administered antiplatelet drugs or anticoagulants, and could discontinue drug treatment for one week before and after ERCP or EST; and (6) patients with a platelet count of 50000/mL or more, and PT-INR 1.5 or less. Exclusion criteria were (1) patients whose medical record could not be reviewed after treatment; and (2) other patients who were judged as inappropriate by the investigator. There were 157 men and 115 women, whose mean age was 74.165 ± 11.649

(44-96) years. The disease was pancreatic cancer in 208 patients, bile duct cancer in 30, metastatic biliary obstruction in 16, duodenal papillary carcinoma in 9, gallbladder cancer in 7, hepatocellular carcinoma in 1, and intraductal papillary mucinous carcinoma in 1 patient. Hepatic metastasis was found in 56 patients and ascites retention in 68 patients. Duodenal stenosis was detected in 40 patients and a duodenal stent was inserted in all patients. In 250 patients the diagnosis of malignancy was established by pathological examination, and in the remaining patients imaging techniques and clinical course. The bile duct diameter prior to drainage was 12.093 ± 3.336 (4-23) mm, and the stenosis length prior to the drainage was 27.276 ± 12.683 (5-59) mm. The result of hepatobiliary enzyme tests prior to the drainage was ALT 141.139 ± 105.309 (29-851) IU/L, ALP 1498.163 ± 1248.051 (409-2620) IU/L, and T-Bil 5.388 ± 6.203 (0.4-27.8) mg/dL. Performance status (PS) determined according to the criteria of the Eastern Cooperative Oncology Group was 0 in 80 patients, 1 in 10 patients, 2 in 56 patients, 3 in 66 patients and 4 in 60 patients. Underlying diseases included high blood pressure in 107 patients, diabetes mellitus in 81 patients, hyperlipidemia in 26 patients, chronic lung disease in 43 patients, heart disease in 38 patients, chronic liver disease in 24 patients, chronic kidney disease in 13 patients, cerebrovascular disease in 25 patients, and dementia in 25 patients; and there were 32 patients who were administered antiplatelet drugs or anticoagulants. Chemotherapy was carried out in 121 patients based on judgment by the attending physician. The judgment of therapeutic effect was made after 2 courses of chemotherapy. In patients for whom chemotherapy could not be continued before conducting 2 courses, the effect was judged at that time. Chemotherapy effect was judged using RECIST criteria[9]. At the time of effect judgment, complete response: CR was defined as disappearance of all target lesions, and regarding lymph node involvement, this was defined as reduction of the shorter diameter to less than 10 mm. Partial response: PR was defined as reduction by more than 30% compared to the sum of total target lesion diameter at the baseline. Stable disease: SD was defined as no reduction corresponding to PR and no progress corresponding to PD. Progressive disease: PD was defined as an increase by 20% or more and an increase in absolute value by 5 mm or more when compared with the sum of the minimum diameter of the target lesion during the course. Patients under 80 years of age were classified into Group A and patients aged 80 or more into Group B. The safety of metallic stent insertion, patency period after metallic stent insertion, obstruction rate, and success rate of metallic stent insertion were examined and compared between the two groups. Early procedural accidents occurring in ERCP-related procedures were evaluated using Cotton's classification[10]. All treatments were carried out with the patient's or patient's family informed consent, and a signed consent form was obtained. This study was conducted with the approval of the ethics committee of each participating facility.

Statistical analysis

Person χ^2 test with Yates correction and Fisher's exact test, when appropriate, were used for statistical analysis of categorical variables. Stent patency and patient survival time were estimated using the Kaplan-Meier method, and the log-rank test was used to access differences between the groups. Data were analyzed using SPSS software version 11 (SPSS, Chicago, IL, United States). Differences with a P value of < 0.05 were considered statistically significant.

RESULTS

The patient background data is shown in Table 1. In Group B there were significantly more patients with high blood pressure, heart disease, cerebrovascular disease, and dementia, as well as more patients taking antiplatelet drugs or anticoagulants. PS tended to be significantly worse in Group B, and fewer patients were subjected to chemotherapy in this group. Other factors did not show a significant difference between Group A and Group B. The results on treatment are shown in Table 2. The success rate of metallic stent insertion was 100% (272/272). The drainage effect after insertion of the metal stent was ALT 70.375 ± 123.860 (10-278) IU/L, ALP 768.400 ± 70.181 (326-1855) IU/L, and T-Bil 1.944 ± 2.873 (0.4-12.2) mg/dL, showing significant reduction ($P < 0.05$). There was no significant difference of ALT, ALP and T- Bil values after the drainage between Group A and Group B, and drainage was excellent. Chemotherapy was conducted more frequently in Group A and best supportive care was more frequently employed in Group B. As for the effect of chemotherapy, CR was achieved in 0% (0/121), PR in 13.2% (16/121), SD in 70.2% (85/121), and PD in 16.5% (20/121) of the patients, with a success rate of 13.2% (16/121). Prior to conducting 2 courses of chemotherapy, PD was observed in 8.3% (10/121) of the patients, thus it was not possible to continue with the treatment. In Group A, the number of patients with SD tended to be significantly higher. The median of survival period was 205.000 ± 21.801 (7-999) d. The median of survival period was 246.000 ± 36.056 (7-738) d in Group A and 180.000 ± 25.724 (7-999) d in Group B, showing no significant difference (Figure 1). The median patency period was 265.000 ± 26.779 (1-965) d; 252.000 ± 35.998 (1-618) d in Group A and 269.000 ± 47.885 (1-965) d in Group B, with no significant difference (Figure 2). Stent obstruction occurred in 30.15% (82/272) of the patients during the follow-up period. The cause of stent obstruction included overgrowth in 6.3% (17/272), ingrowth in 2.2% (6/272), sludge in 9.6% (26/272), migration in 8.5% (23/272), food impaction

Table 1 Patient background

	< 80 yr old (Group A)	⩾ 80 yr old (Group B)	*P* value
Number of patients	184	88	
Male	105	52	NS
Female	79	36	NS
Pancreatic cancer	136	72	NS
Bile duct cancer	21	9	NS
Metastatic biliary obstruction	13	3	NS
Papilla vater cancer	6	3	NS
Gallbladder cancer	6	1	NS
Intraductal papillary mucinous carcinoma	1	0	NS
Hepatocellular carcinoma	1	0	NS
Hepatic metastasis	40	16	NS
Ascites	45	23	NS
Duodenal stent indwelling	30	10	NS
Pathological diagnosis	161	89	NS
Performance status			
0	72	8	0.0000
1	7	3	NS
2	44	12	0.018
3	29	37	0.0004
4	32	28	0.0064
Chemotherapy	106	15	0.0000
Hypertension	55	52	0.0002
Hyperlipidemia	20	6	NS
Diabetes mellitus	50	31	NS
Chronic respiratory disease	25	18	NS
Cardiac disease	18	20	0.0065
Chronic liver disease	18	6	NS
Chronic renal disease	10	3	NS
Cerebrovascular disease	5	20	0.0000
Dementia	5	20	0.0000
Anticoagulant/antiplatelet	9	23	0.0270

NS: Not significant.

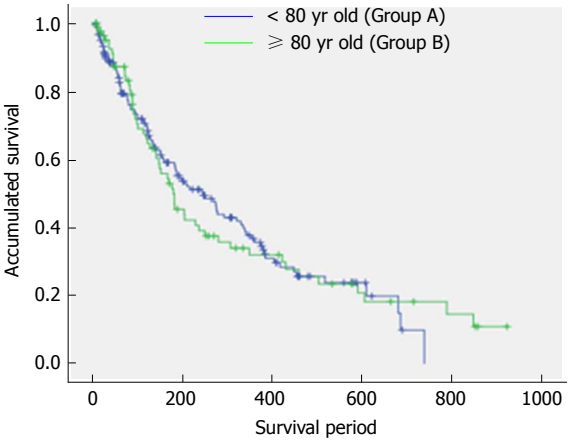

Figure 1 Comparison of the survival period. The median of survival period was 246.000 ± 36.056 (7-738) d in Group A and 180.000 ± 25.724 (7-999) d in Group B, showing no significant difference.

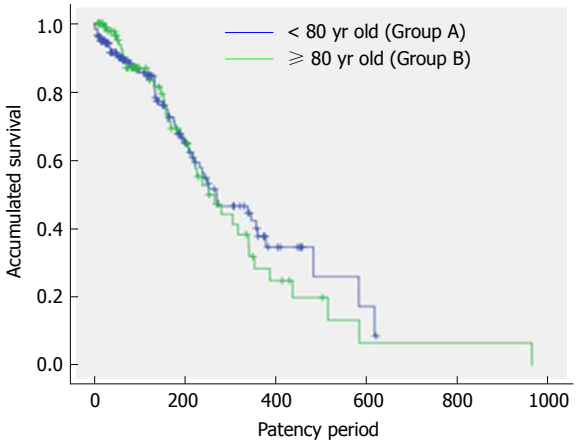

Figure 2 Metallic stent patency period. The median patency period was 265.000 ± 26.779 (1-965) d; 252.000 ± 35.998 (1-618) d in Group A and 269.000 ± 47.885 (1-965) d in Group B, with no significant difference.

in 2.6% (7/272), and others in 1.1% (3/272). Stent obstruction occurred in 28.80% (53/184) of patients in Group A, and in 32.95% (29/88) of those in Group B with no significant difference between the two groups. There was no significant difference between Group A and Group B regarding the cause-specific obstruction rate (Table 3). Early procedural accidents due to ERCP related procedures were observed in 8.8% (24/272) of the patients. Early procedural accidents included pancreatitis in 2.9% (8/272, mild 8), cholecystitis in 3.7% (10/272, mild 8, moderate 2), cholangitis in 1.1% (3/272 mild 3), stent migration in 0.4% (1/272), and pneumonia in 0.7% (2/272). As shown in Table 4, there was no significant difference in the incidence of procedural accidents between Group A and Group B, whereas the onset of pancreatitis showed a

Table 2 Treatment effect judgment

Drainage effect	< 80 yr old (Group A; *n* = 184)	≥ 80 yr old (Group B; *n* = 88)	*P* value
Bile duct diameter prior to drainage (mm)	11.350 ± 3.6310 (4-23)	12.819 ± 3.3148 (5-22)	NS
Stenosis length prior to drainage (mm)	27.216 ± 12.688 (5-59)	27.292 ± 12.702(6-52)	NS
ALT prior to drainage (IU/L)	162.767 ± 104.768 (29-851)	127.567 ± 104.760(30-838)	NS
ALT post drainage (IU/L)	78.875 ± 159.482 (10-278)	66.039 ± 143.106 (13-270)	NS
ALP prior to drainage (IU/L)	1529.375 ± 1465.465 (409-2620)	1489.447 ± 1102.192 (432-2512)	NS
ALP post drainage (IU/L)	871.000 ± 803.131 (326-1855)	665.622 ± 557.643 (331-1811)	NS
T-Bil prior to drainage (mg/dl)	5.438 ± 6.512 (0.4-27.8)	5.276 ± 5.858 (0.6-27)	NS
T-Bil post drainage (mg/dl)	2.387 ± 3.879 (0.4-12.2)	1.580 ± 3.943 (0.6-12)	NS
Chemotherapeutic effect			
CR	0	0	NS
PR	12	4	NS
SD	78	7	0.0000
PD	16	4	NS
Total	106	15	0.0000
Best supportive care	78	73	0.0000

NS: Not significant.

Table 3 Cause of metallic stent obstruction

Related complications	< 80 yr old (Group A; *n* = 184)	≥ 80 yr old (Group B; *n* = 88)	*P* value
Overgrowth	10	7	NS
Ingrowth	6	0	NS
Sludge	16	10	NS
Stent migration	15	8	NS
Food impaction	3	4	NS
Others	3	0	NS
Total	53	29	NS

NS: Not significant.

Table 4 Comparison of early procedural accidents after conducting endoscopic retrograde cholangiopancreatography

Related complications	< 80 yr old (Group A; *n* = 184)	≥ 80 yr old (Group B; *n* = 88)	*P* value
Bleeding	0	0	
Perforation	0	0	NS
Pancreatitis	7 (mild)	1 (mild)	0.003
Cholangitis	1 (mild)	2 (mild)	NS
Cholecystitis	8 (6 mild, 2 moderate)	2 (mild)	NS
Stent migration	1	0	NS
Pneumoniae	0	2	NS

NS: Not significant.

significantly lower tendency in Group B: 0.01% (1/88) when compared with in Group A: 3.8% (7/184). All procedural accidents resolved without requiring a surgical procedure.

DISCUSSION

In medical care treatment should be safer and minimally invasive in consideration of the patient's quality of life. ERCP related procedures can be carried out rather safely as they are minimally invasive and a reasonable treatment method for removal of a calculus from the common bile duct, or drainage in patients with obstructive jaundice. There are many reports indicating that ERCP related procedures can be safely performed even in the elderly[11-14]. However, sufficient attention is necessary because there also are reports describing that procedural accidents easily become serious once they occur[15]. In the present study we assessed the feasibility of metallic stent insertion in elderly patients with unresectable distal malignant biliary obstruction. The study was conducted because there have been no such reports so far. In general, elderly people tend to have various underlying diseases[11-15], and this study also showed that tendency. Although it is obvious that the patient condition is not so good in such a situation, we showed that a metal stent can be safely inserted even in the elderly. In this study, there was no significant difference in the survival period and the metallic stent patency period. In recent years, patient prognosis has improved thanks to advances in chemotherapy for pancreatic cancer and bile duct cancer[16-18]. There is a report showing that not only the survival period but also metal stent patency period is prolonged by chemotherapy for bile duct cancer[19]. However, in this study, no significant difference was found in the survival period and the patency period, though the number of patients who underwent chemotherapy in the elderly group was significantly small. The reason may be that the success rate of chemotherapy was low in this study. The success rate of metal stent insertion was 100% in both groups. Though it is a retrospective study, the success rate of transpapillary metallic stent insertion is generally high even in past reports[5-8]. Therefore, this result seems not to be a particularly good result. There was no significant difference in procedural accidents between the two groups, but the incidence of pancreatitis tended to be lower in Group B. The reason may be that in the elderly, pancreatic function is deteriorated, which lowers the possibility of

developing pancreatitis[20]. In addition, pneumonia was observed in Group B but not in Group A. Since elderly people have sometimes reduced sputum excretion capacity, they may suffer from aspiration pneumonia. Therefore, especially for the elderly it was considered necessary to frequently conduct sputum aspiration during and after surgery. This study suggested that metallic stents can be safely inserted and are a useful treatment for unresectable distal malignant biliary obstruction in the elderly. However, since this was a retrospective, multicenter study, a prospective study is considered necessary in the future to further evaluate the feasibility and usefulness of this procedure in elderly patients.

In conclusion, it was suggested that metallic stent insertion for unresectable distal malignant biliary obstruction in the elderly can be conducted safely and with a high success rate, without any significant difference in the occurrence of procedural accidents when compared with the non-elderly group, even though the elderly tend to have more underlying diseases.

ARTICLE HIGHLIGHTS

Research background

The related procedure of endoscopic retrograde cholangiopancreatography (ERCP) is a minimally invasive diagnostic and therapeutic method for biliary and pancreatic diseases and plays a very important role. As for biliary drainage, endoscopic drainage by ERCP is considered to be noninvasive and the first choice of treatment.

Research motivation

To our knowledge, there are no reports that examined the safety of insertion of metallic stents in the elderly with an unresectable malignant biliary obstruction. Therefore, we studied the safety and usefulness of metallic stents for unresectable distal malignant biliary obstruction in a multicenter, collaborative, retrospective study.

Research objectives

This study aims to evaluate the safety of insertion of metallic stents in elderly patients with unresectable distal malignant biliary obstruction.

Research methods

There are total 272 patients with unresectable distal malignant biliary obstruction in this study. Group A (184 patients under the age of 80) and Group B (88 subjects aged 80 years or more) were examined by the safety of metallic stent insertion, metal stent patency period, and the obstruction rate.

Research results

In Group B, patients had a significantly worse performance status, high blood pressure, cerebrovascular disease, heart disease, and dementia; besides the rate of patients orally administered antiplatelet drugs or anticoagulants tended to be higher ($P < 0.05$). Metallic stents were successfully inserted in all patients. The median patency period was 265.000 ± 26.779 d (1-965); 252.000 ± 35.998 d (1-618) in Group A and 269.000 ± 47.885 d (1-965) in Group B, with no significant difference between the two groups. Metallic stent obstruction occurred in 30.15% (82/272) patients; in 28.80% (53/184) patients in Group A and in 32.95% (29/88) of those in Group B, showing no significant difference between the two groups.

Research conclusions

This study suggested that metallic stents can be safely inserted and are a useful treatment for unresectable distal malignant biliary obstruction in the elderly. It was suggested that metallic stent insertion for unresectable distal malignant biliary obstruction in the elderly can be conducted safely and with a high success rate. We should insert metallic stent in unresectable distal malignant biliary obstruction in the elderly patients for improvement of quality of life positively.

Research perspectives

A prospective study is considered necessary in the future to further evaluate the feasibility and usefulness of metallic stent for unresectable distal malignant biliary obstruction in elderly patients.

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ORIGINAL ARTICLE

Retrospective Study

Short- and long-term outcomes following laparoscopic *vs* open surgery for pathological T4 colorectal cancer: 10 years of experience in a single center

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Abstract

AIM

To evaluate the short-term and long-term outcomes following laparoscopic *vs* open surgery for pathological T4 (pT4) colorectal cancer.

METHODS

We retrospectively analyzed the short- and long-term outcomes of proven pT4 colorectal cancer patients who underwent complete resection by laparoscopic or open surgery from 2006 to 2015 at Guangdong General Hospital.

RESULTS

A total of 211 pT4 colorectal cancer patients were included in this analysis, including 101 cases in the

laparoscopy (LAP) group and 110 cases in the open surgery (OPEN) group [including 15 (12.9%) cases of conversion to open surgery]. Clinical information (age, gender, body mass index, comorbidities, American Society of Anesthesiologists score, *etc.*) did not differ between the two groups. In terms of blood loss, postoperative complications and rate of recovery, the LAP group performed significantly more favorably ($P < 0.05$). With regard to pT4a/b and combined organ resection, there were significantly more cases in the OPEN group ($P < 0.05$). The 3- and 5-year overall survival rates were 74.9% and 60.5%, respectively, for the LAP group and 62.4% and 46.5%, respectively, for the OPEN group ($P = 0.060$). The 3- and 5-year disease-free survival rates were 68.0% and 57.3%, respectively, for the LAP group and 55.8% and 39.8%, respectively, for the OPEN group ($P = 0.053$). Multivariate analysis showed that ⅢB/ⅢC stage, lymph node status, and CA19-9 were significant predictors of overall survival. PT4a/b, ⅢC stage, histological subtypes, CA19-9, and adjuvant chemotherapy were independent factors affecting disease-free survival.

CONCLUSION

Laparoscopy is safely used in the treatment of pT4 colorectal cancer while offering advantages of minimal invasiveness and faster recovery. Laparoscopy is able to achieve good oncologic outcomes similar to those of open surgery. We recommend that laparoscopy be carried out in experienced centers. It is still required to screen the appropriate cases for laparoscopic surgery, optimize the preoperative diagnosis process, and reduce the conversion rate. Multi-center, prospective, and large-sample studies are required to assess these issues.

Key words: pT4 colorectal cancer; Laparoscopy; Open surgery

Core tip: Laparoscopy has been widely used in the treatment of colorectal cancer and has achieved a good radical effect in oncology. However, current clinical association guidelines do not recommend laparoscopic surgery for T4 colorectal cancer. This study retrospectively collected the data of pathological T4 (pT4) colorectal cancer patients at Guangdong General Hospital from 2006 to 2015, aiming to compare outcomes of laparoscopic *vs* open surgery. The conclusion is that laparoscopy is safely used in the treatment of pT4 colorectal cancer while offering advantages of faster recovery. Laparoscopy is able to achieve good oncologic outcomes similar to those of open surgery.

Yang ZF, Wu DQ, Wang JJ, Lv ZJ, Li Y. Short- and long-term outcomes following laparoscopic *vs* open surgery for pathological T4 colorectal cancer: 10 years of experience in a single center. *World J Gastroenterol* 2018; 24(1): 76-86 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i1/76.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i1.76

INTRODUCTION

Colorectal cancer is a common malignant tumor. It is the third most diagnosed cancer and the fourth leading cause of cancer-related deaths worldwide[1]. In China, the incidence and mortality of colorectal cancer are ranked among the top five of all cancers; thus, colorectal cancer is a very serious public health problem[2]. In promoting comprehensive, individualized, and precise treatments to date, surgical treatment is still the only way to cure colorectal cancer. Since 1991, when Jacobs first reported the technical feasibility of laparoscopic colectomy[3], a number of successful randomized controlled studies have been conducted around the world to compare laparoscopy and laparotomy, with encouraging results achieved. The laparoscopic treatment of colorectal cancer can not only achieve similar short- and long-term outcomes comparable to laparotomy, but its advantage of minimal invasiveness has gradually been recognized and promoted[4-7]. The American Joint Committee on Cancer (AJCC) classifies T4 colorectal cancers as those that invade into other organs and structures and/or perforate the visceral peritoneum; laparoscopic surgery in colorectal cancer at this stage is difficult as it is hard to reach and violates the "no touch" principle. Therefore, the AJCC and European Association of Endoscopic Surgery do not recommend laparoscopic treatment of pathological T4 (pT4) colorectal cancer[8]. This study retrospectively collected the data of pT4 colorectal cancer patients at Guangdong General Hospital from 2006 to 2015, aiming to compare the outcomes of laparoscopic *vs* open surgery.

MATERIALS AND METHODS

Patients

All pT4 colorectal cancer patients treated at Guangdong General Hospital from 2006 to 2015 were enrolled in this study. All patients were staged according to the AJCC 7th edition manual for colorectal cancer. The inclusion criteria included the following: (1) age of 18-75 years; (2) proven T4 pathology; and (3) radical surgery (D3 lymph node dissection). The exclusion criteria included the following: (1) low rectal cancer (peritoneal reflection as the boundary); (2) preoperative neoadjuvant treatment; (3) non-neoplastic deaths; and (4) palliative resection.

Surgical procedure

Preoperative computed tomography (CT) and magnetic resonance imaging were used to determine the

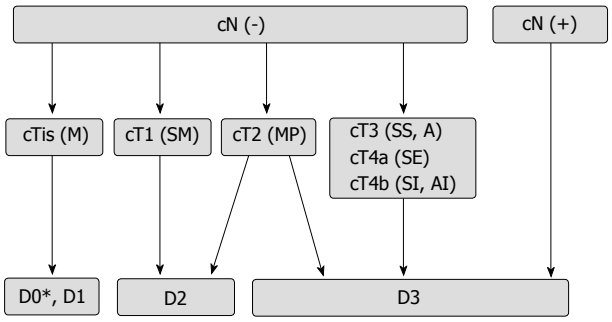

Figure 1 Flowchart for selection of the extent of lymph node dissection (from reference 9).

preoperative clinical stage. The decision to proceed with laparoscopy or open surgery was made for all subjects on a patient-by-patient basis following multidisciplinary discussions and meetings. All cases entailed surgical resection according to the Japanese Society for Cancer of the Colon and Rectum (JSCCR) guidelines[9], which have the following requirements: D3 lymph node dissection (Figure 1) to ensure the appropriate resection length, and ensuring that the integrity of the mesorectum and intraoperative operations follow the principle of "no touch" (sharp separation, blood vessels first, tumor isolation, *etc.*). According to tumor location, the method of resection included the following: total colectomy, right colectomy, extended right colectomy, transverse colectomy, left colectomy, sigmoid colectomy, mid/upper anterior resection, and combined organ resection. Laparoscopic incision should not exceed 6 cm. The conversion cases were analyzed in the open surgery (OPEN) group.

Observation indexes

The preoperative indexes included age, gender, body mass index (BMI; kg/m^2), comorbidity, American Society of Anesthesiologists (ASA) score, tumor location, hemoglobin, and tumor markers (CA19-9 and CEA). The intraoperative indexes included surgical and pathological outcomes. Surgical outcomes included the conversion rate (conversion was defined as an open surgery performed during the laparoscopic procedure in order to ensure complete resection, reconstruction, or hemostasis and not just for the extraction of specimens), tumor size, resection length, operative time, blood loss, intraoperative complications, combined organ resection, postoperative complications and mortality. Pathological outcomes included the number of lymph nodes dissected, lymph node status, margin, pT stage, pN stage, pTNM stage, Dukes stage, histological subtype, and differentiation. The postoperative recovery indexes included time to flatus, diet, and ambulation and hospital stays.

Follow-up

All patients were postoperatively referred to the 7th AJCC/UICC TNM stage for adjuvant chemotherapy. All patients were followed through outpatient visits. According to the NCCN guidelines, patients were subjected to a 5-year surveillance program consisting of physical examinations and tumor marker (CEA and CA19-9) analysis every 3 mo up to 2 years. Every 6 mo, patients had complete colonoscopies at one and three years after surgery. Thoracic and abdominal CT scans were planned every year for five years of surveillance.

Statistical analysis

Statistical analyses were performed using SPSS 19.0. Quantitative data are reported as the mean ± SD or median. Categorical data were compared by χ^2 tests or Fisher's exact test. Survival curves [overall survival (OS) and disease-free survival (DFS)] were derived from Kaplan-Meier estimates, and the curves were compared by the log-rank test. Prognostic factors were identified by univariate analysis and further tested by multivariate analysis. The results are reported as a hazard ratio (HR) with 95%CI. A P-value < 0.05 was considered statistically significant.

RESULTS

During the period from 2006 to 2015, we collected a total of 211 pT4a/bN0-2M0 cases according to enrollment criteria from 2308 cases of colorectal cancer at the Department of General Surgery of Guangdong General Hospital. There were 101 cases in the laparoscopy (LAP) group and 111 cases in the OPEN group (Figure 2).

There were no significant differences in age, gender, BMI, ASA score, tumor location, hemoglobin, CA19-9, or CEA between the two groups (P > 0.05) (Table 1).

For surgical outcome, conversion to open surgery occurred in 15 (12.9%) patients, and all conversion cases were analyzed in the OPEN group. There was no significant difference between the two groups in terms of intraoperative complications and postoperative complications within 30 d (P > 0.05). Laparoscopic surgery was slightly slower than open surgery (210.8 ± 88.9 min *vs* 173.5 ± 72.7 min, P = 0.028); there was less blood loss (155.0 ± 75.9 mL *vs* 235.1 ± 120.5 mL, P = 0.033) in laparoscopic surgery, whereas open surgery showed better resection lengths (15.5 ± 7.3 cm *vs* 19.5 ± 10.4 cm, P = 0.046). In the case of combined organ resection, there were 21 (19.1%) patients in the OPEN group, including three cases of abdominal wall resection, five cases of small bowel (except duodenum) resection, three cases of duodenum resection, two cases of urinary organ resection, one case of stomach resection, four cases of gynecologic organ resection, and three cases of liver resection; in contrast, there were only five cases in the

Table 1 Clinical information of 211 colorectal cancer cases

Clinical information		LAP *n* = 101	OPEN *n* = 110	*P* value
Age	> 60 yr	55	58	0.270
	⩽ 60 yr	46	52	
Gender	Male	67	66	0.392
	Female	34	44	
BMI (kg/m^2)	< 24	67	73	0.348
	⩾ 24	34	37	
Comorbidities	Yes	39	42	1.000
	No	62	68	
ASA score	I	8	9	0.715
	II	63	72	
	III	30	29	
Tumor location	Mid/upper Rectum	33	35	0.989
	Left colon	43	47	
	Right colon	25	28	
HBG (g/L)	mean ± SD	124.0 ± 27.1	120.7 ± 22.9	0.263
CA19-9 (U/mL)	< 27	78	75	0.163
	⩾ 27	23	35	
CEA (ng/mL)	< 5	60	64	0.666
	⩾ 5	41	46	
Postoperative adjuvant chemotherapy	Yes	49	46	0.332
	No	52	64	
Recurrence	Yes	22	25	0.711
	No	79	85	

CRC: Colorectal cancer; LAP: Laparoscopic surgery group; OPEN: Open surgery group; BMI: Body mass index; ASA: American Society of Anesthesiology; HGB: Hemoglobin; CA19-9: Carbohydrate antigen 19-9; CEA: Caicinoembryonic antigen; SD: Standard deviation.

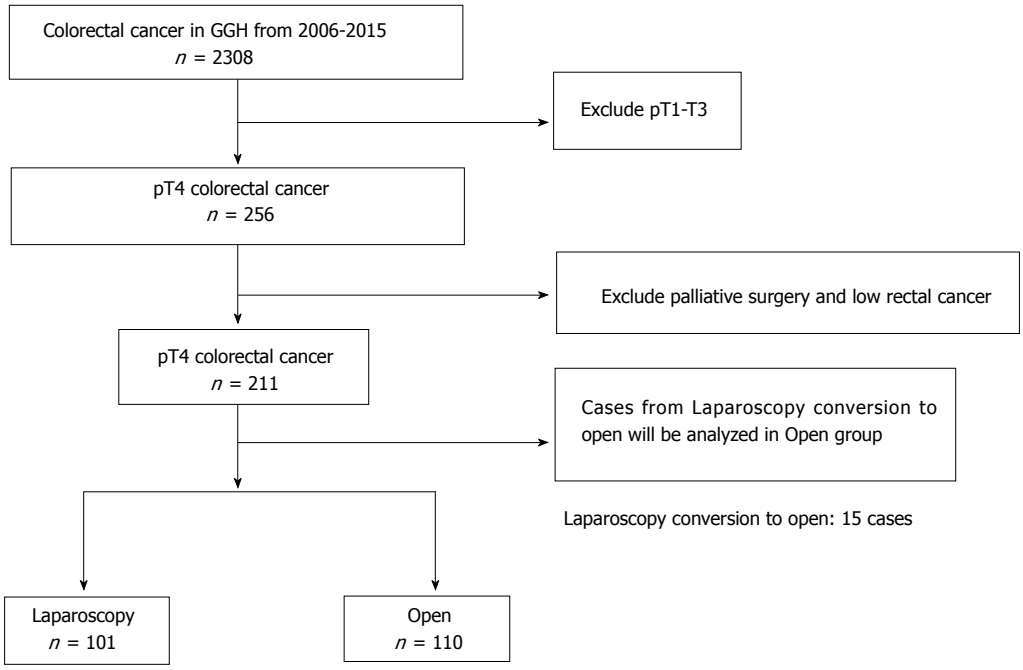

Figure 2 Study flowchart showing patient selection. GGH: Guangdong general hospital; pT4: Pathological proven T4.

LAP group (P = 0.001). For postoperative complications within 30 d, there were 12 (12.9%) cases in the LAP group, and there was a higher incidence in the OPEN group (31.8%, P = 0.006) (Table 2).

Regarding pathologic outcomes, no significant differences in the number of lymph nodes dissected, lymph node status, margin, pN stage, pTNM stage, Dukes stage, histological subtype, differentiation, or HER2 status were detected when comparing the two groups (P > 0.05). There were 21 pT4b cases in the OPEN group but only five cases in the LAP group; a comparison between the two groups in the pT stage revealed a statistically significant difference (P = 0.021) (Table 3).

Table 2 Surgical outcomes of 211 colorectal cancer cases

Surgical outcome		LAP *n* = 101	OPEN *n* = 110	*P* value
Conversion to open (*n*/%)		15 (12.9)	NR	/
Tumor size (cm)	mean ± SD	5.4 ± 1.9	5.2 ± 2.5	0.765
Resection length (cm)	mean ± SD	15.5 ± 7.3	19.5 ± 10.4	0.046
Operative time (min)	mean ± SD	210.8 ± 88.9	173.5 ± 72.7	0.028
Blood loss (mL)	mean ± SD	155.0 ± 75.9	235.1 ± 120.5	0.033
Intraoperative complications		3	8	0.117
Combined organ resection	Total (%)	5 (5.0)	21 (19.1)	0.001
	Abdominal wall	2	3	
	Small bowel (except duodenum)	1	5	
	Duodenum	0	3	
	Urinary organs	0	2	
	Stomach	1	1	
	Gynecologic organs	1	4	
	Liver	0	3	
Postoperative complications within 30 d	Total (%)	12 (12.9)	35 (31.8)	0.006
	Anastomotic Hemorrhage	1	2	
	Urinary injury	0	1	
	Intraabdominal bleeding	1	2	
	Leakage	1	4	
	Gastroplegia	2	4	
	Infection (incision and abdomen)	6	15	
	Disruption of incision	0	5	
	Obstruction	1	2	
Postoperative morbidity within 30 d		0	1	0.667

CRC: Colorectal cancer; LAP: Laparoscopic surgery group; OPEN: Open surgery group.

Table 3 Pathologic outcomes of 211 colorectal cancer cases

Pathologic outcome		LAP *n* = 101	OPEN *n* = 110	*P* value
Number of lymph nodes dissected	< 12	25	38	0.134
	⩾ 12	76	72	
Lymph node status	+	67	74	1.000
	-	34	36	
Margin	R1	2	3	0.779
	R0	99	107	
pT stage	T4a	96	89	0.021
	T4b	5	21	
pN stage	N0	36	35	0.841
	N1	28	32	
	N2	37	43	
pTNM stage	ⅡB + ⅡC	34	35	0.282
	ⅢB	30	24	
	ⅢC	37	51	
Dukes	B	34	35	0.883
	C	67	75	
Histological subtype	Adenocarcinoma	87	94	1.000
	Myxoadenocarcinoma	14	16	
Differentiation	Poor	20	25	0.719
	Median/high	81	85	
HER2	-/+	86	96	0.871
	++	10	10	
	+++	5	4	

CRC: Colorectal cancer; LAP: Laparoscopic surgery group; OPEN: Open surgery group; SD: Standard deviation; p: Pathological.

With regard to postoperative recovery indexes, the LAP group was significantly better than the OPEN group ($P < 0.05$) in time to flatus, diet, and ambulation. The median hospital stay was 7 (5-21) d for the LAP group and 15 (7-31) d for the OPEN group, which showed a statistically significant difference between the two groups ($P = 0.004$) (Table 4).

The mean overall follow-up time was 36 mo

Table 4 Postoperative recovery outcomes of 211 colorectal cancer cases

Recovery outcome		LAP *n* = 101	OPEN *n* = 110	*P* value
Time to flatus (d)	Median (range)	2 (1-9)	4 (3-15)	0.037
Time to diet (d)	Median (range)	3 (2-18)	7 (5-27)	0.003
Time to ambulation (d)	Median (range)	2 (1-5)	5 (3-9)	0.027
Hospital stays	Median (range)	7 (5-21)	15 (7-31)	0.004

LAP: Laparoscopic surgery group; OPEN: Open surgery group.

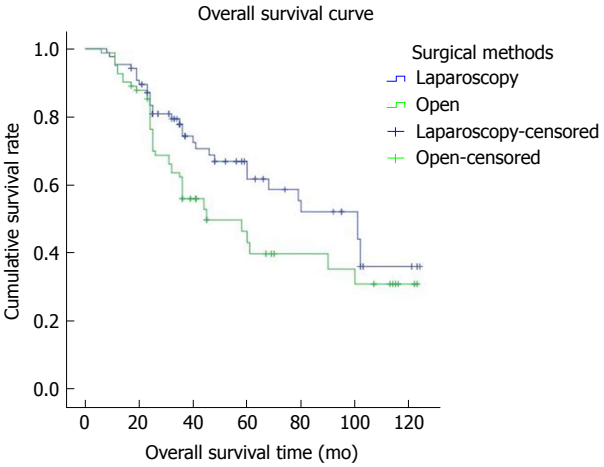

Figure 3 The overall survival curve shows that 3- and 5-year overall survival rates were 74.9% and 60.5%, respectively, in the LAP group and 62.4% and 46.5%, respectively, in the OPEN group. There was no significant difference between the LAP and OPEN groups (P = 0.060).

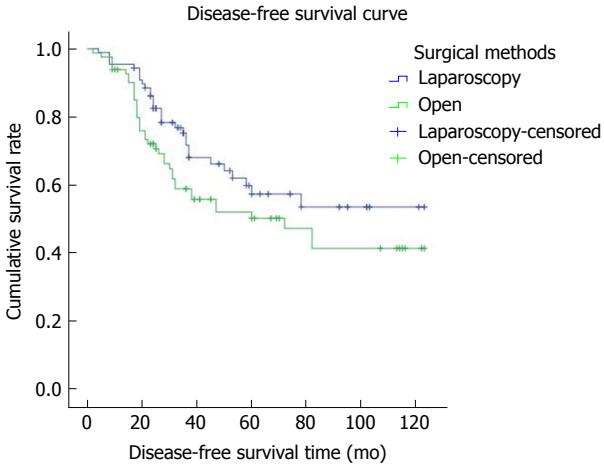

Figure 4 The disease-free survival curve shows that the 3- and 5-year disease-free survival rates were 68.0% and 57.3%, respectively, in the LAP group and 55.8% and 39.8%, respectively, in the OPEN group. There was no significant difference between the LAP and OPEN groups (P = 0.053).

(range, 2-24 mo); there was no difference between the LAP and OPEN groups in terms of OS and DFS. The 3- and 5-year OS rates were 74.9% and 60.5%, respectively, for the LAP group and 62.4% and 46.5%, respectively, for the OPEN group (P = 0.60) (Figure 3). The 3- and 5-year DFS rates were 68.0% and 57.3%, respectively, for the LAP group and 55.8 and 39.8%, respectively, for the OPEN group (P = 0.053) (Figure 4). Disease recurrence over the entire follow-up period was observed in 21.8% (n = 22) of patients in the LAP group and 22.7% (n = 25) of patients in the OPEN group (P = 0.711) (Table 1), without significant differences between the LAP and OPEN groups (P = 0.711). In the multivariate regression analysis, TNM stage (ⅢB, ⅢC), lymph node status (pN+), and CA19-9 were significant predictors of OS. TNM stage (ⅢC), histological subtype, CA19-9, and chemotherapy were predictive of DFS (Tables 5 and 6).

DISCUSSION

Since the first report of laparoscopic colorectal resection in 1991, some prospective clinical studies of laparoscopic resection for colorectal cancer have confirmed that laparoscopic techniques not only achieve minimally invasive and cosmetic effects but also achieve faster recovery and similar oncologic outcomes compared with open surgery[10-12]. However, due to the large tumor size of T4 colorectal cancer and more frequent invasion of peripheral tissues or nearby organs, laparoscopic complete resection is difficult and has high risks; the majority of clinical studies have fewer cases of T4 colorectal cancer[13,14], and some studies do not enroll any such cases[15,16]. Therefore, the evidence-based data that support the laparoscopic resection in T4 colorectal cancer are limited. Laparoscopic resection of T4 colorectal cancer is regarded a technique that demands precision, and its efficacy remains controversial. The relevant guidelines do not recommend laparoscopy in T4 colorectal cancer[8]. However, due to the maturity and progress of the laparoscopic platform, coupled with the popularity of and improvements in laparoscopic techniques, some surgeons in certain experienced centers have tried to use laparoscopic techniques in T4 colorectal cancer, achieving similar short- and long-term outcomes to open surgery[17-20].

This study decided whether laparoscopic or open surgery should be performed based on the results of preoperative imaging examination and the patient's condition; the main referenced indicators included the following: tumor location, tumor size, and the scope of invaded organ[21]. We found a statistically

Table 5 Univariate and multivariate analyses of 211 pathological T4 colorectal cancer patients for overall survival

	Univariate analysis		Multivariate analysis	
Variable	**HR (95%CI)**	***P* value**	**HR (95%CI)**	***P* value**
Age	1.217 (0.784-1.888)	0.381		
Gender	0.807 (0.508-1.281)	0.363		
Surgical method (LAP and OPEN)	1.528 (0.982-2.377)	0.060		
Tumor location				
Mid/upper rectum	Reference group	-		
Left colon	1.303 (0.813-2.091)	0.272		
Right colon	0.792 (0.409-1.533)	0.489		
Comorbidities	1.603 (1.007-2.552)	0.047	2.519 (1.436-4.419)	0.142
pT4a/b	0.790 (0.692-1.236)	0.445		
N stage				
N0	Reference group	-		
N1	1.328 (0.701-2.517)	0.384		
N2	2.079 (1.170-3.697)	0.013		
TNM stage				
ⅡB + ⅡC	Reference group	-	Reference group	-
ⅢB	1.229 (0.564-2.679)	0.604	1.324 (0.785-1.753)	0.019
ⅢC	3.092 (1.617-5.913)	0.001	1.104 (0.333-3.662)	0.001
Lymph node status	0.560 (0.324-0.968)	0.038	0.307 (0.103-0.919)	0.035
No. of lymph nodes resected	0.593 (0.385-0.915)	0.018	0.432 (0.264-0.708)	0.123
Histological subtype	0.369 (0.212-0.640)	0.000	0.433 (0.218-0.859)	0.247
Differentiation	0.326 (0.204-0.519)	0.000	0.460 (0.273-0.775)	0.087
CA19-9	1.868 (1.195-2.922)	0.006	1.662 (1.212-2.280)	0.002
CEA	1.089 (0.706-1.680)	0.013	0.608 (0.356-1.038)	0.068
Chemotherapy	1.611 (1.040-2.494)	0.033	2.225 (1.394-3.552)	0.181

CRC: Colorectal cancer; LAP: Laparoscopy surgery; OPEN: Open surgery; p: Pathological; CA19-9: Carbohydrate antigen 19-9; CEA: Carcinoembryonic antigen; HR: Hazard ratio.

Table 6 Univariate and multivariate analyses of 211 pathological T4 colorectal cancer patients for disease-free survival

	Univariate analysis		Multivariate analysis	
Variable	**HR (95%CI)**	***P* value**	**HR (95%CI)**	***P* value**
Age	1.621 (1.010-2.601)	0.045	1.892 (1.111-3.223)	0.419
Gender	1.328 (0.824-2.141)	0.243		
Surgical method (LAP and OPEN)	1.503 (0.933-2.422)	0.094		
Tumor location				
Mid/upper rectum	Reference group	-		
Left colon	1.010 (0.601-10697)	0.969		
Right colon	0.818 (0.411-1.629)	0.568		
Comorbidities	1.787 (1.058-3.019)	0.030	2.261 (1.235-4.139)	0.080
pT4a/b	0.818 (0.618-1.725)	0.013	1.214 (0.784-1.974)	0.001
N stage				
N0	Reference group	-		
N1	1.134 (0.594-2.167)	0.703		
N2	1.553 (0.861-2.801)	0.144		
TNM stage				
ⅡB + ⅡC	Reference group	-	Reference group	-
ⅢB	1.034 (0.471-2.269)	0.933	0.884 (0.393-1.989)	0.765
ⅢC	2.284 (1.202-4.337)	0.012	1.831 (0.935-3.584)	0.018
Lymph node status	0.710 (0.411-1.229)	0.221		
No. of lymph nodes resected	0.661 (0.411-1.061)	0.087		
Histological subtype	0.456 (0.243-0.854)	0.014	0.469 (0.225-0.974)	0.042
Differentiation	0.439 (0.266-0.725)	0.001	0.662 (0.374-1.170)	0.156
CA19-9	2.458 (1.526-3.960)	0.000	3.372 (1.968-5.778)	0.000
CEA	1.268 (0.790-2.036)	0.326	0.608 (0.356-1.038)	0.072
Chemotherapy	2.157 (1.323-3.514)	0.002	3.817 (2.194-6.639)	0.000

CRC: Colorectal cancer; LAP: Laparoscopic surgery; OPEN: Open surgery; p-Pathological; CA19-9: Carbohydrate antigen 19-9; CEA: Carcinoembryonic antigen; HR: Hazard ratio.

Table 7 Studies about laparoscopic surgery for pathological T4 colorectal cancer

Ref.	*n* (LAP:OPEN)	Study period	Study design	Country	Single/Multi center	Conversion rate	Location	Complication rate (LAP:OPEN)	Tumor stage	Combined resection (LAP:OPEN)	RI rate (LAP:OPEN)	5 years DFS (LAP:OPEN)	5 years OS (LAP:OPEN)
Park *et al*[22], 2016	93:18	2000-2010	RS	South Korea	Single	5.6%	Colon rectum	14.1%:31.5%	-	9.9%:32.5%	5.5%:4.5%	81.8%:73.9%	95.3%:86.5%
Shukla *et al*[20], 2015	61:22	2003-2011	RS	United States	Single	21%	Colon	28%:36%	Ⅱ:35 Ⅲ:48	23%:41%	0%:4%	75%:65% (3 yr)	82%:81% (3 yr)
Kang *et al*[29], 2016	52:57	2003-2013	RS	South Korea	Single	7.7%	Colon	13.5%:36.8%	Ⅱ:41 Ⅲ:68	13.5%:36.8%	-	53.6%:62.6%	60.7%:61.9%
de'Angelis *et al*[18], 2016	106:106	2005-2014	RPSM	France Switzerland	Multi	12.2%	Colon	29.1%:35.3%	Ⅱ:85 Ⅲ:127	14.2%:18.9%	5.7%:6.6%	58.6%:59.9%	57.6%:50.2%
de'Angelis *et al*[17], 2016	52:52	2005-2015	RPSM	France Switzerland Spain	Multi	21.2%	Rectum	30.8%:48.1%	Ⅱ:42 Ⅲ:33 Ⅳ:29	26.9%:30.8%	19.2%:17.3%	66.7%:64.1%	55.4%:53.3%
Chan *et al*[30], 2016	93:59	2008-2014	RS	Singapore	Single	8.6%	Colon	-	-	0%:3.4%	0%:0.7%	-	75%:80%
Leon *et al*[27], 2017	68:79	2008-2015	RS	Italy	Single	19%	Colon	7.4%:16.5%	Ⅱ:69 Ⅲ:78	-	11.8%:11.5%	40.3%:38.9%	44.6%:39.4%
Ahmad *et al*[25], 2015	455:406	2011-2012	RS	Canada	ACSNS QIP	24.7%	Colon	-	-	-	26.2%:24.3%	-	-

LAP: Laparoscopy; RS: Retrospective study; RPSM: Retrospective propensity score matching; ACSNS: American College of Surgeons National Surgical Quality Improvement Program; OS: Overall survival; DFS: Disease-free survival.

significant difference in the postoperative pT stage between the two groups (P = 0.021), with 21 cases of pT4b in the OPEN group. An examination of postoperative surgical outcomes (Table 2) revealed 21 cases of combined organ resection in the OPEN group, with the most commonly invaded organs including the small intestine, gynecological organs, and duodenum; in contrast, the LAP group had only five cases, and the number of cases of combined organ resection was thus significantly different for the two groups (P = 0.001), which is consistent with the results of previous studies[22,23]. These data also demonstrate that T4b stage may be an important consideration for surgeons to select laparoscopic or open surgery because it is very difficult to achieve the goal of complete resection using the "no touch" principle; therefore, guidelines do not recommend laparoscopic resection in T4 colorectal cancer[8]. However, such considerations lead to selective bias in the study, which is one of the limitations in both this study and a retrospective study.

Because of the larger tumor, a wide scope of invasion, combined with resection of other organs, especially due to the lack of laparoscopic experience in some centers, may lead to a high conversion rate. Previous studies of laparoscopic surgery in colorectal cancer (stage Ⅱ-Ⅲ)[11,15,24] reported that the conversion rate was 25% in the CLASSIC trial (for colon cancer), 17% in the COLOR trial, and 21% in the COSTSG trial, whereas a retrospective study of pT4 colorectal cancer showed that the conversion rate was 5.6%-24.7%[17,19,22,25-29]; this study showed a conversion rate of 12.9%, which is consistent with reports in the literature. The conversion rates reported by some studies of pT4 colorectal cancer in South Korea and Singapore were 5.6%, 7.7% and 8.6%[22,29,30], which are significantly lower than those from the US and Europe[17,18,25,27] (Table 7). We explain these differences as follows: (1) Laparoscopic technology and experience may differ between Asian and Western countries; (2) the conversion standard used was different; (3) there was a lack of preoperative imaging assessments to select appropriate laparoscopic surgery cases in Western countries; and (4) European populations had a higher BMI. All these factors increase the difficulty of surgery[17-25]. Thus, surgeons should choose the appropriate pT4 colorectal cases to perform laparoscopic surgery in order to reduce the conversion rate and ensure operation safety. We recommend laparoscopic surgery as an option in experienced centers and for T4a cases with tumor sizes < 5 cm and when only a single organ has been invaded by T4 colorectal cancer.

In this study, the LAP group had longer operative time (210.8 ± 88.9 min *vs* 173.5 ± 72.7 min, P = 0.028), which may be related to the lack of experience and

the difficulty of this surgery[19]. The resection length (19.5 ± 10.4 cm) obtained in the OPEN group was significantly better (P = 0.046). The literature shows that the incidence of postoperative complications associated with laparoscopic surgery was clearly lower than that associated with open surgery[31]. In this study, the rate of postoperative complication within 30 d in the LAP group was 12.9% (12/101), which was lower than 31.8% (35/110) in the OPEN group, and the most common complications in the OPEN group were infection (incision and abdomen) and disruption of incision, similar to a previous report in the literature[32]. Therefore, attention should be paid to intraoperative sterile principles and the suture of incision.

With regard to postoperative recovery outcomes, the LAP group had clear advantages in time to flatus (P = 0.037), diet (P = 0.003), and ambulation (P = 0.027) and hospital stays (P = 0.004) compared with the OPEN group (Table 4). Laparoscopy has the advantages of minimal invasion and fast recovery, which is in agreement with many earlier clinical studies[12,15,16].

In colorectal cancer surgery, lymph node dissection and R0 resection are important factors affecting long-term survival[33]. We performed D3 lymphadenectomy (parenteral lymph node -middle lymph node-central lymph node)[34] according to the guidelines recommended by the JSCCR. Previous studies have shown that laparoscopic treatment of colorectal cancer achieved an R0 resection rate between 80.8% and 98%[11,16,18]. In this study, the percentage of cases with the number of lymph nodes dissected greater than 12 was 75.2% (76/101) in the LAP group and 65.5% (72/110) in the OPEN group, and the difference was not significantly different (P = 0.134). Concurrently, the R0 resection rate in the LAP group was 98% (99/101), whereas in the OPEN group, it was 97.3% (107/110), which was not significantly different (P = 0.779). Thus, we believe that laparoscopic treatment in pT4 colorectal cancer can achieve similar oncological outcomes to open surgery. Finally, no differences in the 3- and 5-year OS rates (P = 0.060) and in 3- and 5-year DFS rates (P = 0.053) were observed when comparing the two groups, suggesting that laparoscopy may be a valid and effective tool to treat pT4 colorectal cancer without jeopardizing oncologic results, in accordance with the previously reported series. Multivariate analysis in our series detected ⅢB/ⅢC stage, lymph node status, and CA19-9 as independent predictors of OS, and pT4a/b, ⅢC stage, CA19-9, and adjuvant chemotherapy as independent predictors of DFS.

In conclusion, laparoscopic surgery may be safe and acceptable in the treatment of pathologic T4 colorectal cancer patients with fast recovery outcomes and oncologic outcomes compared with open surgery. Thus, laparoscopy should not be regarded as an absolute contraindication in the management of pT4 colorectal cancer. Finally, as this study is only a retrospective study in a single center with a small sample size, the results need to be confirmed by prospective, multi-center and large sample clinical studies.

ARTICLE HIGHLIGHTS

Research background

Laparoscopy has been widely used in the treatment of colorectal cancer and it has achieved a good radical effect in oncology. However, for the current clinical guidelines, laparoscopic surgery is not recommended in T4 colorectal cancer.

Research motivation

Due to the characteristics of T4 colorectal cancer, laparoscopic complete resection is difficult for the resection of this kind of tumor. The current colorectal studies about laparoscopy have fewer cases of T4 colorectal cancer, and some studies do not enroll any such cases. We tried to collect and analyze the data about laparoscopy in T4 colorectal cancer in order to add evidence-based clinical evidence.

Research objectives

We aimed to analyze the short- and long-term outcomes of proven pathological T4 colorectal cancer patients who underwent complete resection by laparoscopic or open surgery.

Research methods

We collected and analyzed the data of pT4 colorectal cancer cases at Guangdong General Hospital from 2006 to 2015. All patients were staged according to the AJCC 7th edition manual for colorectal cancer. We compared the laparoscopy (LAP) group and open (OPEN) group in clinical information, surgical and pathological outcomes, postoperative recovery outcomes, and survival.

Research results

There were 101 cases in the LAP group and 110 cases in the OPEN group [including15 (12.9%) cases of conversion to open surgery]. Clinical information did not differ between the two groups. In terms of blood loss, postoperative complications, and rate of recovery, the LAP group performed significantly more favorably (P < 0.05). With regard to pT4a/b and combined organ resection, there were significantly more cases in the OPEN group (P < 0.05). The 3- and 5-year overall survival rates were 74.9% and 60.5%, respectively, for the LAP group and 62.4% and 46.5%, respectively, for the OPEN group (P = 0.060). The 3- and 5-year disease-free survival rates were 68.0% and 57.3%, respectively, for the LAP group and 55.8% and 39.8%, respectively, for the OPEN group (P = 0.053). Multivariate analysis showed that ⅢB/ⅢC stage, lymph node status, and CA19-9 were significant predictors of overall survival. PT4a/b, ⅢC stage, histological subtype, CA19-9, and adjuvant chemotherapy were independent factors affecting disease-free survival.

Research conclusions

Laparoscopic surgery may be safe and acceptable in the treatment of pathologic T4 colorectal cancer patients with fast recovery outcomes and oncologic outcomes compared with open surgery. We recommend that it can be carried out in experienced centers. It is required to screen the appropriate cases for laparoscopic surgery, optimize the preoperative diagnosis process, and reduce the conversion rate.

Research perspectives

Although our study shows that laparoscopy is able to achieve good clinicopathological and oncologic outcomes similar to those of open surgery, this study is only a retrospective study in a single center with a small sample, and the results need to be confirmed by prospective, multi-center and large sample clinical studies.

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ORIGINAL ARTICLE

Retrospective Study

Differential analysis of lymph node metastasis in histological mixed-type early gastric carcinoma in the mucosa and submucosa

Qian Zhong, Qi Sun, Gui-Fang Xu, Xiu-Qin Fan, Yuan-Yuan Xu, Fei Liu, Shi-Yi Song, Chun-Yan Peng, Lei Wang

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Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymized clinical data that were obtained after each patient agreed by written consent to undergo curative gastrectomy with lymph node dissection.

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Abstract

AIM

To investigate the relationship between histological mixed-type of early gastric cancer (EGC) in the mucosa and submucosa and lymph node metastasis (LNM).

METHODS

This study included 298 patients who underwent gastrectomy for EGC between 2005 and 2012. Enrolled lesions were divided into groups of pure differentiated (pure D), pure undifferentiated (pure U), and mixed-

type according to the proportion of the differentiated and undifferentiated components observed under a microscope. We reviewed the clinicopathological features, including age, sex, location, size, gross type, lymphovascular invasion, ulceration, and LNM, among the three groups. Furthermore, we evaluated the predictors of LNM in the mucosa-confined EGC.

RESULTS

Of the 298 patients, 165 (55.4%) had mucosa-confined EGC and 133 (44.6%) had submucosa-invasive EGC. Only 13 (7.9%) cases of mucosa-confined EGC and 30 (22.6%) cases of submucosa-invasive EGC were observed to have LNM. The submucosal invasion (OR = 4.58, 95%CI: 1.23-16.97, *P* = 0.023), pure U type (OR = 4.97, 95%CI: 1.21-20.39, *P* = 0.026), and mixed-type (OR = 5.84, 95%CI: 1.05-32.61, *P* = 0.044) were independent risk factors for LNM in EGC. The rate of LNM in mucosa-confined EGC was higher in the mixed-type group (*P* = 0.012) and pure U group (*P* = 0.010) than in the pure D group, but no significant difference was found between the mixed-type group and pure U group (*P* = 0.739). Similarly, the rate of LNM in the submucosa-invasive EGC was higher in the mixed-type (*P* = 0.012) and pure U group (*P* = 0.009) than in the pure D group but was not significantly different between the mixed-type and pure U group (*P* = 0.375). Multivariate logistic analysis showed that only female sex (OR = 5.83, 95%CI: 1.64-20.70, *P* = 0.028) and presence of lymphovascular invasion (OR = 13.18, 95%CI: 1.39-125.30, *P* = 0.020) were independent risk factors for LNM in mucosa-confined EGC, while histological type was not an independent risk factor for LNM in mucosa-confined EGC (*P* = 0.106).

CONCLUSION

For mucosal EGC, histological mixed-type is not an independent risk factor for LNM and could be managed in the same way as the undifferentiated type.

Key words: Early gastric carcinoma; Mixed-type; Lymph node metastasis; Mucosa; Lymphovascular invasion

Core tip: This retrospective study investigated the relationship between the histological mixed-type of early gastric carcinoma (EGC) in the mucosa and submucosa and lymph node metastasis (LNM). We found that the rates of LNM in the histological mixed-type and the pure undifferentiated type were not significantly different in the mucosal or submucosal EGC. Furthermore, histological type was not an independent risk factor for LNM in mucosa-confined EGC. Hence, according to WHO classification, histological mixed-type and pure undifferentiated EGC could be managed in the same way, and curative endoscopic submucosal dissection is feasible for patients with histological mixed-type mucosa-confined EGC.

Zhong Q, Sun Q, Xu GF, Fan XQ, Xu YY, Liu F, Song SY, Peng CY, Wang L. Differential analysis of lymph node metastasis in histological mixed-type early gastric carcinoma in the mucosa and submucosa. *World J Gastroenterol* 2018; 24(1): 87-95 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i1/87.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i1.87

INTRODUCTION

Gastric carcinoma is the second leading cause of cancer-related death behind lung carcinoma, despite a worldwide decline in both its incidence and mortality since the late half of the twentieth century[1]. Endoscopic resection can be curative for selected cases of early gastric cancer (EGC)[2]. Endoscopic submucosal dissection (ESD), a recently developed technique, is a widely accepted treatment modality for EGC, including undifferentiated-type EGC within the expanded criteria (mucosal cancer measuring less than 2 cm without ulceration or lymphovascular tumor emboli). Some studies have shown that there is no risk of lymph node metastasis (LNM) in undifferentiated-type EGC, conforming to the expanded criteria for endoscopic resection[3-5]. The incidence of LNM is the most important factor when deciding on endoscopic resection in ECG[6]. Therefore, the criteria for ESD consist of factors related to the risk of LNM, including histological differentiation and tumor size.

The prognosis of EGC is mainly dependent on histological differentiation, which determines the extent of LNM and can potentially assist in selecting the most suitable treatment strategy[7-9]. However, gastric cancer tissues often present histological heterogeneity and comprise a mixture of several different cell types. Before a treatment has been selected, it is difficult for pathologists to accurately diagnose the histological type of the cancer tissue based on the histological differentiation state definitions as defined by the 14th Japanese classification of gastric carcinoma[10] or the 7th tumor-node-metastasis classification[11]. This is especially the case in mixed-type gastric cancer from several biopsy specimens. Approximately 5%-25% of gastric cancers are classified as having a histological mixed-type in previous studies[12,13], consisting of undifferentiated and differentiated components.

Recently, several studies have reported that mixed-type EGC was associated with aggressive clinical features as well as poor outcomes[12,14]. However, these studies did not consider the different biological behaviors of the mucosal mixed-type EGC and submucosal mixed-type EGC, although patients with submucosal EGC would undoubtedly be at a higher risk of LNM and poor prognosis than those with mucosal EGC. Therefore, the biological behavior of the histological mixed-type in mucosal EGC remains undetermined. In this study, we aimed to clarify the relationship of histological mixed-type EGC and the rate of LNM, and the feasibility of

endoscopic resection for patients with mixed-type EGC in the mucosa.

MATERIALS AND METHODS

Patients

A consecutive series of 298 patients diagnosed with EGC were examined. All patients, including 165 patients with EGC in the mucosa and 133 patients with EGC in the submucosa, underwent curative gastrectomy with lymph node dissection at the Department of Surgery at Nanjing Drum Tower Hospital from January 2005 to January 2012. Patients who were treated with chemotherapy before surgery were excluded from this study. Histopathological examination was performed by expert pathologists. Data on the clinicopathological factors, including age, sex, tumor location, macroscopic appearance, size, presence of stomach ulcer, infection with *Helicobacter pylori* (*H. pylori*), lymphovascular invasion, and presence of LNM, were obtained. After surgery, patients were told to visit our outpatient department for esophagogastroduodenoscopy and abdominal pelvic computed tomography, which were performed at 3- and 6-mo intervals during the first year and then annually thereafter. This study was approved by the Institutional Review Board of Nanjing Drum Tower Hospital.

Subgroups based on the histological differentiation state

Patients were divided into three subgroups according to the histological features of their tumors: (1) a pure differentiated component with no undifferentiated component (pure D group); (2) a pure undifferentiated component with no differentiated component (pure U group); and (3) a histological mixed-type that consisted of both differentiated and undifferentiated components (mixed-type group). The histological mixed-type includes the differentiated-predominant mixed-type with undifferentiated component making up less 50% (D > U group) and the undifferentiated-predominant mixed-type with undifferentiated component making up more than 50% (U > D group). According to the Japanese classification of gastric carcinoma, the pure D and D > U groups were classified as the differentiated type of gastric cancer, whereas the U > D and pure U groups were classified as the undifferentiated type. On the other hand, according to the tumor-node-metastasis classification, only the pure D group was classified as the differentiated type, and the remaining three groups were classified as the undifferentiated type. The Japanese classification of gastric carcinoma[6] was used to classify the macroscopic type of the tumors as follows: elevated (Ⅰ, Ⅱa, Ⅰ and Ⅱa, Ⅱa and Ⅱb), flat (Ⅱb), and depressed (Ⅱc, Ⅱc and Ⅲ, Ⅲ). According to the different histological classifications, the cases of EGC in the mucosa and submucosa are shown in Figure 1.

Statistical analysis

Analysis of variance was used to compare the mean values of the continuous variables among the three different histological subgroups, and the χ^2 test was performed on the categorical variables. Bonferroni correction for multiple comparisons suggested differences between two of the three histological subgroups. Multivariate logistic regression analysis was performed to determine the predictors of LNM. The significant difference was set at an alpha level of 0.05. When the Bonferroni correction was applied, the significant difference was set at an alpha level of 0.017, which was 0.05/number of tests[15]. All statistical analyses were performed using SPSS software, version 22.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

Clinicopathological characteristics of patients with EGC

The study group consisted of 206 male patients and 92 female patients with a median age of 59.5 years (range, 18-86 years). Seventy-one patients had tumors in the upper third of the stomach and 227 in the middle or lower third. One hundred and six patients had an elevated gross type and 192 patients had a flat or depressed gross type. There were 165 patients with EGC in the mucosa and 133 patients with EGC in the submucosa. Histologically, patients were divided into the following four groups: 188 (63.1%) patients with the pure D histological type, 24 (7.0%) with the D > U histological type, 17 (5.7%) with the U > D histological type, and 69 (23.2%) with the pure U histological type. The overall prevalence of the histological mixed-type, including the D > U and U > D histological types, was 12.7% (41/298) in EGC. The overall incidence of LNM was 14.4% in EGC (Table 1). In a univariate analysis of EGC, LNM was associated with younger age (P = 0.005), female sex (P = 0.044), tumor size (P = 0.022), middle/lower location (P = 0.010), lymphovascular invasion (P = 0.013), flat/depressed gross type (P = 0.034), depth of submucosal invasion (P = 0.001), pure U type (P = 0.005), and mixed-type (P = 0.001). Given that the criteria for ESD treatment of EGC consist of the invasion depth, histological type, tumor size, and ulceration, it is reasonable and meaningful to take these four covariates into a multivariate analysis to assess their main effect on the rate of LNM. The multivariate analysis showed that submucosal invasion (OR = 4.58, 95%CI: 1.23-16.97, P = 0.023), pure U type (OR = 4.97, 95%CI: 1.21-20.39, P = 0.026), and mixed-type (OR = 5.84, 95%CI: 1.05-32.61, P = 0.044) were independent risk factors for LNM in EGC. There was no interaction effect between the invasion depth and histological type (P = 0.822) (Table 2).

Clinicopathologic features among the histological mixed-type, pure undifferentiated, and pure differentiated EGC in the mucosa and submucosa

Table 3 shows the relationship between the clinico-

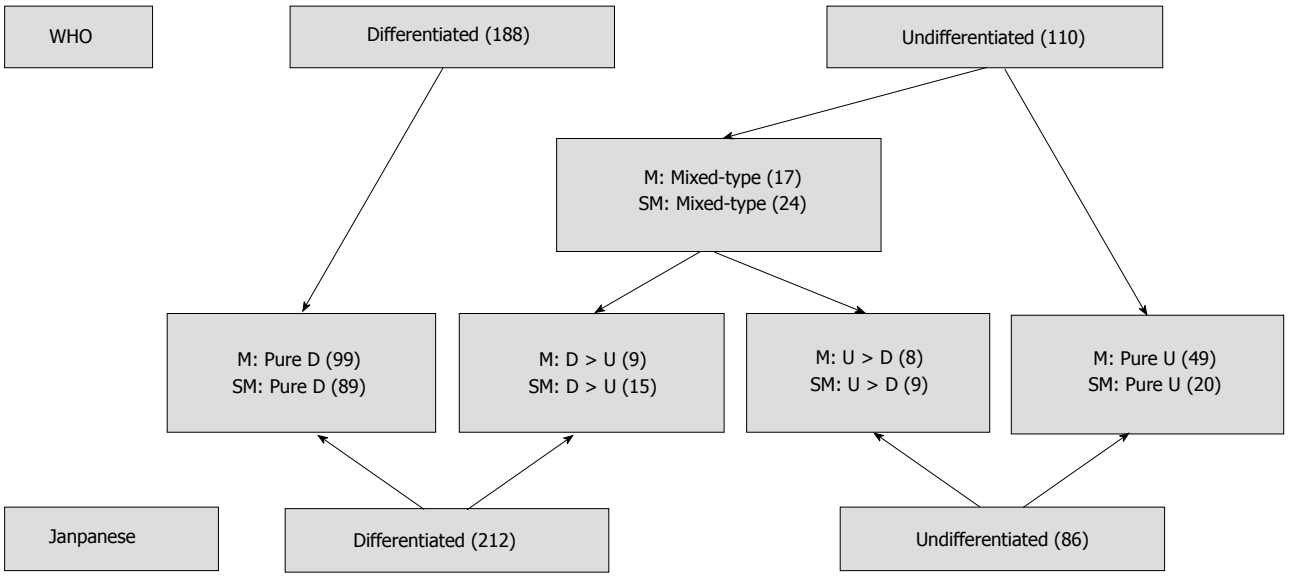

Figure 1 The relationship among the histological classifications of early gastric cancer in the mucosa and submucosa. Of the 298 cases, there were 212 with differentiated type gastric cancer and 86 with undifferentiated type gastric cancer based on the Japanese classification currently used as the endoscopic resection criteria. In accordance with the WHO classification, there were 188 patients with differentiated type and 110 patients with undifferentiated type gastric cancer. According to the histological differentiation and undifferentiation components, 41 patients were reclassified as mixed-type. For mucosal early gastric cancer (EGC), there were 99 patients in the pure differentiated (pure D) group, 9 in the differentiated > undifferentiated (D > U) group, 8 in the undifferentiated > differentiated (U > D) group, and 49 in the pure undifferentiated (pure U) group. For submucosal EGC, there were 89 in the pure D group, 15 in the D > U group, 9 in the U > D group, and 20 in the pure U group. Among the 41 patients with mixed-type EGC, there were 17 in the mucosa and 24 in the submucosa. D: Differentiated; M: Mucosa; SM: Submucosa; U: Undifferentiated.

Table 1 Clinicopathological characteristics of patients with early gastric cancer *n* (%)

Variable	Value
Age (yr), median ± SD (range)	59.5 ± 12.1 (18-86)
Gender	
Male	206 (69.1)
Female	92 (30.9)
Tumor size (cm), median ± SD (range)	2.2 ± 1.2 (0.5-6.0)
Location	
U	71 (23.8)
M/L	227 (76.2)
Gross type	
Elevated	106 (35.6)
Flat/depressed	192 (64.4)
Depth of invasion	
M	165 (55.4)
SM	133 (44.6)
Histological type	
Pure D	188 (63.1)
D > U	24 (7.0)
U > D	17 (5.7)
Pure U	69 (23.2)
Lymph node metastasis	
Present	43 (14.4)
Absent	255 (85.6)

SD: Standard deviation; U: Upper; M/L: Middle/lower; M: Mucosa; SM: Submucosa; Pure D: Pure differentiated; D: Differentiated components; U: Undifferentiated components; Pure U: Pure undifferentiated.

pathological factors and histological types, which included the mixed-type, pure undifferentiated type, and pure differentiated type, in mucosal EGC. The distribution of age (P = 0.028), gender (P = 0.004), tumor location (P = 0.003), gross type (P < 0.001), ulceration (P = 0.015), *H. pylori* (P = 0.025), and LNM (P = 0.016) significantly differed among the three histological subgroups, while tumor size (P = 0.802) and lymphovascular invasion (P = 0.589) were similarly distributed among the three histological subgroups. Patients in the mixed-type group were more likely to have *H. pylori* infection than those in the pure U group (P = 0.015). Besides tumor size and lymphovascular invasion, age (P = 0.525), gender (P = 0.151), tumor location (P = 0.759), gross type (P = 0.507), and ulceration (P = 0.490) were similarly distributed between the mixed-type group and pure U group. Of note, the rate of LNM was similar between the mixed-type group and the pure U group (P = 0.739), although it was significantly higher in the mixed-type group (P = 0.012) and pure U group (P = 0.010) than in the pure D group. These data suggested that mixed-type group showed similar clinicopathologic features and aggressive behavior compared with the pure U group in mucosal EGC.

Table 4 shows the relationship between the clinicopathological factors and histological types, which included the mixed type, pure undifferentiated type, and pure differentiated type in submucosal EGC. Distribution of age (P = 0.008), gender (P < 0.001), tumor location (P = 0.019), gross type (P = 0.028), and LNM (P = 0.008) significantly differed among the three histological subgroups, while tumor size (P = 0.166), ulceration (P = 0.369), *H. pylori* infection (P = 0.997), and lymphovascular invasion (P = 0.325)

Table 2 Risk factors for lymph node metastasis in early gastric carcinoma

Variable	Univariate analysis			Multivariate analysis		
	OR	95%CI	*P* value	OR	95%CI	*P* value
Age (yr)			0.005			
⩽ 60	1.00					
> 60	0.37	0.18-0.74				
Gender			0.044			
Male	1.00					
Female	1.97	1.02-3.82				
Size (cm)	1.33	1.04-1.71	0.022			0.154
Location			0.010			
U	1.00					
M/L	4.85	1.45-16.19				
LV invasion			< 0.001			
Absent	1.00					
Present	18.4	8.31-40.72				
Gross type			0.034			
Elevated	1.00					
Flat/depressed	2.32	1.07-5.04				
Ulcer			0.031			0.256
Absent	1.00					
Present	2.15	1.07-4.31				
H. pylori infection			0.346			
Absent	1.00					
Present	1.46	0.67-3.19				
Depth of invasion			0.001			0.023
Mucosa	1.00					
Submucosa	3.41	1.70-6.83		4.58	1.23-16.97	
Histological type			0.001			0.054
Pure D	1.00					
Pure U	2.99	1.39-6.44	0.005	4.97	1.21-20.39	0.026
Mixed-type	4.45	1.91-10.36	0.001	5.84	1.05-32.61	0.044
Depth of invasion/histological type						0.822

MT: Mixed-type; PU: Pure undifferentiated; PD: Pure differentiated; U: Upper; M/L: Middle/lower; LV: Lymphovascular; LNM: Lymph node metastasis.

Table 3 Clinicopathologic features of mucosa-confined early gastric carcinoma according to histological type *n* (%)

Variable	MT, *n* = 17	Pure U, *n* = 49	Pure D, *n* = 99	*P* value	*P* (Bonferroni corrected)
Age (yr)				0.028	MT *vs* PU, 0.525
⩽ 60	10 (58.8)	33 (67.3)	44 (44.4)		MT *vs* PD, 0.272
> 60	7 (41.2)	16 (32.7)	55 (55.6)		PU *vs* PD, 0.009
Gender				0.004	MT *vs* PU, 0.151
Male	7 (41.2)	30 (61.2)	77 (77.8)		MT *vs* PD, 0.002
Female	10 (58.8)	19 (38.8)	22 (22.2)		PU *vs* PD, 0.034
Tumor size (cm)	2.1 ± 1.1	1.9 ± 1.2	1.9 ± 1.2	0.802	
Location				0.003	MT *vs* PU, 0.759
U	1 (5.9)	4 (8.2)	29 (29.3)		MT *vs* PD, 0.042
M/L	16 (94.1)	45 (91.8)	70 (70.7)		PU *vs* PD, 0.004
Gross type				< 0.001	MT *vs* PU, 0.507
Elevated	4 (23.5)	8 (16.3)	51 (51.5)		MT *vs* PD, 0.033
Flat/depressed	13 (76.5)	41 (83.7)	48 (48.5)		PU *vs* PD, < 0.001
Ulcer				0.015	MT *vs* PU, 0.490
Absent	6 (35.3)	22 (44.9)	64 (64.6)		MT *vs* PD, 0.022
Present	11 (64.7)	27 (55.1)	35 (35.4)		PU *vs* PD, 0.022
H. pylori infection				0.025	MT *vs* PU, 0.015
Absent	1 (5.9)	18 (36.7)	22 (22.2)		MT *vs* PD, 0.119
Present	16 (94.1)	31 (63.3)	77 (77.8)		PU *vs* PD, 0.061
LV invasion				0.589	
Absent	17 (100.0)	47 (95.9)	97 (98.0)		
Present	0 (0)	2 (4.1)	2 (2.0)		
LNM				0.016	MT *vs* PU, 0.739
Absent	14 (82.4)	42 (85.7)	96 (97.0)		MT *vs* PD, 0.012
Present	3 (17.6)	7 (14.3)	3 (3.0)		PU *vs* PD, 0.010

MT: Mixed-type; PU: Pure undifferentiated; PD: Pure differentiated; U: Upper; M/L: Middle/lower; LV: Lymphovascular; LNM: Lymph node metastasis.

Table 4 Clinicopathologic features of submucosa-invasive early gastric carcinoma according to histological type *n* (%)

Variable	MT, *n* = 24	Pure U, *n* = 20	Pure D, *n* = 89	*P* value	*P* (Bonferroni corrected)
Age (yr)				0.008	MT *vs* PU, 0.423
≤ 60	14 (58.3)	14 (70.0)	32 (36.0)		MT *vs* PD, 0.048
> 60	10 (41.7)	6 (30.0)	57 (64.0)		PU *vs* PD, 0.005
Gender				< 0.001	MT *vs* PU, 0.069
Male	15 (62.5)	7 (35.0)	70 (78.7)		MT *vs* PD, 0.104
Female	9 (37.5)	13 (65.0)	19 (21.3)		PU *vs* PD, < 0.001
Size (cm)	2.6 ± 1.3	2.8 ± 1.4	2.3 ± 1.1	0.166	
Location				0.019	MT *vs* PU, 0.128
U	5 (20.8)	1 (5.0)	31 (34.8)		MT *vs* PD, 0.191
M/L	19 (79.2)	19 (95.0)	58 (65.2)		PU *vs* PD, 0.008
Gross type				0.028	MT *vs* PU, 0.199
Elevated	6 (25.0)	2 (10.0)	35 (39.3)		MT *vs* PD, 0.195
Flat/depressed	18 (75.0)	18 (90.0)	54 (60.7)		PU *vs* PD, 0.012
Ulcer				0.369	
Absent	5 (20.8)	7 (35.0)	32 (36.0)		
Present	19 (79.2)	13 (65.0)	57 (64.0)		
H. pylori infection				0.997	
Absent	7 (29.2)	6 (30.0)	26 (29.2)		
Present	17 (70.8)	14 (70.0)	63 (70.8)		
LV invasion				0.325	
Absent	15 (62.5)	15 (75.0)	69 (77.5)		
Present	9 (37.5)	5 (25.0)	20 (22.5)		
LNM				0.008	MT *vs* PU, 0.375
Absent	15 (62.5)	12 (60.0)	76 (85.4)		MT *vs* PD, 0.012
Present	9 (37.5)	8 (40.0)	13 (14.6)		PU *vs* PD, 0.009

MT: Mixed-type; PU: Pure undifferentiated; PD: Pure differentiated; U: Upper; M/L: Middle/lower; LV: Lymphovascular; LNM: Lymph node metastasis.

were similarly distributed among the three histological subgroups. Besides tumor size, ulceration, *H. pylori* infection, and lymphovascular invasion, age (P = 0.423), gender (P = 0.069), tumor location (P = 0.128), and gross type (P = 0.199) were similarly distributed between the mixed-type group and pure U group. The rate of LNM was higher in the mixed-type group (P = 0.012) and pure U group (P = 0.009) than in the pure D group, but was not significantly different between the mixed-type and pure U group (P = 0.375). These data suggested that the mixed-type group showed similar clinicopathologic features and aggressive behavior compared with the pure U group in submucosal EGC.

Risk factors for LNM in EGC in the mucosa

In a univariate analysis of mucosa-confined EGC, LNM was associated with pure undifferentiated type (P = 0.019), mixed-type (P = 0.026), female sex (P = 0.005), and presence of lymphovascular invasion (P = 0.013). However, in a multivariate analysis, only female sex (OR = 5.83, 95%CI: 1.64-20.70, P = 0.028) and presence of lymphovascular invasion (OR = 13.18, 95%CI: 1.39-125.30, P = 0.020) were independent risk factors for LNM of mucosa-confined EGC, while histological type was not an independent risk factor for LNM of mucosa-confined EGC (P = 0.106) (Table 5).

DISCUSSION

Endoscopic resection is a curative modality for EGC and its indications have been expanded[16,17]. However, histological mixed-type is not categorized in the present criteria for ESD. In this study, we found that submucosal invasion, pure U type, and mixed-type were independent risk factors for LNM in EGC. However, the distribution of tumor size, location, gross type, ulceration, lymphovascular invasion, and LNM rate were similar between the mixed-type group and pure U group in mucosa-confined EGC, and histological mixed-type was not an independent risk factor for LNM in mucosal EGC.

EGC generally shows greater histological diversity than other types of cancer. Even tumors confined to the mucosa show histologic diversity, which tends to increase with deeper invasion and increased tumor diameter[18]. In addition, histological mixed-type has been reported to have a higher LNM rate and more aggressive behavior than other histological types[19]. New difficulties in determining the appropriate management for histological mixed-type EGC arose as a result of improvements in ESD, which allows *en bloc* resection of large superficial gastric lesions and precise histopathological evaluation[20]. In fact, there are two distinct groups of differentiated and undifferentiated mixed-type EGC. The first group shows both differentiated and undifferentiated histological components in the mucosa. The second group shows differentiated components confined in the mucosa and undifferentiated components confined in the submucosa. We hypothesized that these two groups had different prognoses and should be evaluated and managed independently. In our study, we found

Table 5 Risk factors for lymph node metastasis in mucosa-confined early gastric carcinoma

Variable	Univariate analysis			Multivariate analysis		
	OR	95%CI	*P* value	OR	95%CI	*P* value
Age (yr)			0.082			
≤ 60	1.00					
> 60	0.31	0.08-1.16				
Size (cm)	1.36	0.90-2.06	0.145			
Gender			0.005			0.028
Male	1.00			1.00		
Female	5.89	1.72-20.17		5.83	1.64-20.70	
Location			0.630			
U	1.00					
M/L	1.47	0.31-6.95				
LV invasion			0.013			0.020
Absent	1.00			1.00		
Present	13.64	1.75-106.28		13.18	1.39-125.30	
Gross type			0.253			
Elevated	1.00					
Flat/depressed	2.17	0.58-8.22				
Ulcer			0.199			
Absent	1.00					
Present	2.14	0.67-6.85				
H. pylori infection			0.244			
Absent	1.00					
Present	0.50	0.15-1.61				
Histological type			0.035			0.106
Pure D	1.00					
Pure U	5.33	1.32-21.63	0.019	4.46	0.99-20.00	0.051
Mixed-type	6.86	1.26-37.77	0.026	5.31	0.86-33.01	0.073

PD: Pure differentiated; PU: Pure undifferentiated; U: Upper; M/L: Middle/lower; LV: Lymphovascular.

that submucosal invasion, pure U type, and mixed-type were independent risk factors for LNM in EGC. We next analyzed the clinicopathologic features of mucosal and submucosal EGC according to the histological type. The mixed-type group and pure U group showed similar clinicopathologic features and aggressive behavior in mucosal EGC, including the distribution of age, gender, tumor size, location, gross type, ulceration, lymphovascular invasion, and LNM rate. Furthermore, we validated that histological type was not an independent risk factor for LNM in mucosal EGC. Given the mucosal undifferentiated EGC has been included in the expanded criteria for ESD, we recommended that mucosal mixed-type EGC could be treated with ESD. For submucosal EGC, the mixed-type group also showed similar clinicopathologic features and aggressive behavior compared with the pure U group. Submucosal undifferentiated EGC has not been included in the criteria for ESD, due to the high frequency of LNM. Several studies have shown that mixed-type EGC with submucosal invasion carries a high risk of LNM, and endoscopic surgery should be limited to the differentiated type of invasive submucosal EGC without histological heterogeneity[20,21]. Our data supported that the LNM rate of submucosal EGC was significantly higher in the mixed-type group (9/24) and pure U group (8/20) than in the pure D group (13/89). In accordance with the 7th tumor-node-metastasis classification, the mixed-type group and pure U group, including undifferentiated components, were all undifferentiated EGC[22]. Hence, the decision criterion of histological mixed-type in tumor-node-metastasis classification is better than Japanese classification[23].

A study from Shimizu *et al*[23] showed that there was no significant difference in the clinicopathological factors between the D > U and U > D groups[23]. This means that the mixture of differentiated and undifferentiated components contributes to a worse prognosis, no matter the ratio of undifferentiated components to differentiated components. Actually, the pathogenesis and aggressiveness of mixed histology are unclear. Zheng *et al*[24] suggested that the mixed-type components might originate from stem cells with similar genetic traits, but follow different histogenic pathways. Park *et al*[25] found that mixed-type gastric carcinoma frequently showed an enhanced CpG island hypermethylation status, implicating enhanced CpG island promoter hypermethylation in the histogenesis of mixed-type carcinoma. Studies of the mucin phenotype have reported that some cases of differentiated gastric cancer with a gastric phenotype are transformed into undifferentiated gastric cancer during tumor growth and development, increasing the risk of LNM[26]. Although mixed-type carcinomas are thought to be more aggressive in some studies according to the Lauren classification[24,27], a recent study reported that mixed histology, according to WHO classification, showed no LNM within the criteria for endoscopic resection[13]. Our data supported that the rate of LNM was not significantly different in the mixed-type and

pure undifferentiated EGC confined in the mucosa. Limited by the small sample sizes, our data showed considerably wide confidence interval of the OR ratio of lymphovascular invasion, in the univariate and multivariate analyses of mucosal EGC. Although similar data were reported in some studies[28,29], it should be confirmed by additional clinical research with larger sample sizes.

In conclusion, our results demonstrated that the histological mixed-type showed a similar low rate of LNM compared with the pure undifferentiated type in mucosa-confined EGC, and histological type was not an independent risk factor for LNM in mucosa-confined EGC. According to the tumor-node-metastasis classification, mucosal histological mixed-type and undifferentiated EGC could be managed in the same way, and curative ESD is feasible for patients with histological mixed-type EGC confined in the mucosa.

ARTICLE HIGHLIGHTS

Research background

Endoscopic submucosal dissection (ESD) is a curative modality for mucosal-confined early gastric cancer (EGC) and its indications have been expanded. However, EGC tissues often present histological heterogeneity and comprise a mixture of several different cell types. The present criteria for ESD does not take consideration of the histological mixed-type EGC. The rate of lymph node metastasis (LNM) is the most important factor when deciding on endoscopic resection in ECG. Therefore, there is a current pressing need to clarify the relationship of histological mixed-type and the rate of LNM, and the feasibility of endoscopic resection for patients with histological mixed-type EGC confined in the mucosa.

Research motivation

According to the latest research, histological mixed-type EGC was associated with aggressive clinical features as well as poor outcomes, but these studies did not consider the different biological behaviors of the mucosal mixed-type EGC and submucosal mixed-type EGC. Given the mucosal undifferentiated EGC measuring less than 2 cm without ulceration has been included in the expanded criteria for ESD treatment, it is necessary to assess the feasibility of endoscopic resection for patients with histological mixed-type EGC confined in the mucosa.

Research objectives

In this study, we investigated the clinicopathologic features of EGC according to histological type classification to evaluate the biological behavior of mixed-type EGC and the predictive value of histological type on the rate of LNM in mucosa-confined EGC.

Research methods

This study included 298 patients who underwent gastrectomy for EGC between 2005 and 2012. Enrolled lesions were divided into groups of pure differentiated (pure D), pure undifferentiated (pure U), and mixed-type according to the proportion of the differentiated and undifferentiated components observed under a microscope. We reviewed the clinicopathological features, including age, sex, location, size, gross type, lymphovascular invasion, ulceration, and LNM, among the three groups. Furthermore, we evaluated the risk factors for LNM in mucosal EGC.

Research results

Submucosal invasion, pure U type, and mixed-type were independent risk factors for LNM in EGC. The rate of LNM in mucosa-confined EGC was higher in the mixed-type group and pure U group than in the pure D group, but no significant difference was found between the mixed-type group and pure U group. Similarly, the rate of LNM in the submucosa-invasive EGC was higher in the mixed-type and pure U group than in the pure D group, but was not significantly different between the mixed-type and pure U group. Multivariate logistic analysis showed that histological type was not an independent risk factor for LNM in mucosa-confined EGC.

Research conclusions

The distribution of tumor size, location, gross type, ulceration, lymphovascular invasion, and LNM rate were similar between the mixed-type group and pure undifferentiated group in mucosa-confined EGC, and the histological mixed-type was not an independent predictor of LNM in mucosa-confined EGC. According to the tumor-node-metastasis classification, the histological mixed-type and undifferentiated EGC could be managed in the same way, and curative ESD was feasible for patients with mucosal histological mixed-type EGC.

Research perspectives

Endoscopic resection is a curative modality for EGC and its indications have been expanded. Our study indicated that the mucosal histological mixed-type EGC could be managed with curative ESD. With the gradual understanding of the pathogenesis and biological behavior of mixed-type EGC, more patients with mixed-type EGC would benefit from the ESD treatment.

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ORIGINAL ARTICLE

Observational Study

HLA-DQ: Celiac disease *vs* inflammatory bowel disease

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Abstract

AIM

To determine the genetic predisposition to celiac

disease (CeD) in inflammatory bowel disease (IBD) patients by quantifying the frequency of CeD-related human leucocyte antigen (HLA) (HLA-CeD: HLA-DQ2 and -DQ8) in IBD patients globally, by type of IBD and gender, and by calculating the protective/risk contribution of these haplotypes in the development of the IBD disease.

METHODS

We conducted a prospective study with IBD patients from our Unit. Clinical information was gathered and blood was tested for HLA-CeD. The control group was made up of unrelated Valencian organ donors.

RESULTS

1034 subjects were analyzed: 457 IBD [207 ulcerative coliti (UC) and 250 Crohn's disease (CD)] patients and 577 healthy controls. 39% of the controls and 34% of the patients had HLA-CeD (P = 0.0852). HLA-DQ2 was less frequent in UC patients (P = 0.0287), and HLA-DQ8 in CD (P = 0.0217). In women with UC, the frequency of DQ2.5cis (DQB1*02:01-DQA1*05:01) was reduced $\geqslant$ 50% [P = 0.0344; preventive fraction (PF) = 13%]. PFs (7%-14%) were obtained with all HLA-CeD haplotypes. HLA DQB1*02:02-DQA1*02:01 (HLA-DQ2.2) was more frequent in CD patients with respect to controls (P = 0.001) and UC patients (etiological fraction = 15%).

CONCLUSION

HLA-CeD is not more frequent in IBD patients, with an even lower frequency of HLA-DQ2 and -DQ8 in UC and CD respectively. HLA-DQ2.5 confers protection from the development of UC, especially in women, and HLA-DQ8 does so for the appearance of CD. HLA-DQ2.2 is present in 34% of the CD patients and may constitute a genetic risk factor for CD development.

Key words: Genetic predisposition; Celiac disease; Inflammatory bowel disease; Crohn's disease; Human leucocyte antigen; Ulcerative colitis

Core tip: The higher risk for celiac disease (CeD) in inflammatory bowel disease (IBD) is controversial. Since the involvement of human leucocyte antigen (HLA)-DQ2 and -DQ8 antigens (HLA-CeD) in the susceptibility to CeD is clearly established and it has been accepted as a useful test to exclude CeD, we determined the frequency of HLA-CeD in IBD patients. We observed that HLA-CeD is not more frequent in IBD patients, with an even lower frequency of HLA-DQ2 and -DQ8 in ulcerative colitis and Crohn's disease respectively. On the other hand, HLA-DQ2.2 was present in 34% of the Crohn's disease patients and may constitute a genetic risk factor.

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INTRODUCTION

Celiac disease (CeD) and inflammatory bowel disease (IBD) are chronic intestinal disorders with progressively increasing incidences and prevalences[1-11]. Both diseases are thought to be secondary to the interaction of certain environmental factors which either directly cause or enable others to trigger the disease (gluten -cause of CeD-, infections, dysbiosis, *etc.*), in genetically predisposed patients, by producing an altered immunological response.

CeD is a life-long inflammatory condition of the small intestine represented by a gluten-sensitive enteropathy in genetically susceptible individuals[7]. CeD has defined diagnostic criteria[8], which include blood antibodies, genetic testing, upper endoscopy findings and, especially, histological small-bowel changes. The involvement of human leucocyte antigen (HLA) genes codifying HLA-DQ2 and -DQ8 antigens in the susceptibility to the disease is clearly established and HLA typing has been accepted as a useful test to exclude CeD, because only 0.5% of CeD patients lack both DQ2 and DQ8 antigens[9].

Genetic predisposition to CeD, associated to heterodimers HLA-DQ2, encoded by DQB1*02/DQA1*05 alleles [*cis*-encoded in DQB1*02:01-DQA1*05:01 haplotypes (HLA-DQ2.5*cis*) or *trans*-encoded in DQB1*02:02-DQA1*02:01 + DQB1*03:01-DQA1*05:05 genotypes (HLA-DQ2.2 + HLA-DQ7.5: HLA-DQ2.5*trans*)] and, to a lesser degree, HLA-DQ8, encoded by DQB1*03:02/DQA1*03 alleles, has been found to have a high negative predictive value[12,13]. The HLA-DQ2.2 heterodimer has binding properties that are similar to those of HLA-DQ2.5, but it is not considered to predispose for CeD unless it is expressed with the HLA-DQ2.5 or -DQ7.5 heterodimers[9,10].

CeD is more prevalent in women, with a ratio of 2:1 with respect to men, theoretically due to HLA inheritance[14].

IBD patients have historically been considered to be at higher risk for CeD[12], which could be supported by the fact that IBD and CeD are quite prevalent and due to a theoretically similar pathogenesis[11,13,15], with the interaction of genetic, immunological, and environmental factors (gut flora, gastroenteritis, *etc.*).

Several studies have tried to relate IBD and CeD with different results[1,16,17]. None of the studies analyzed the genetic predisposition, although Leeds *et al* suggested that a reduced frequency of HLA-DQ2 and -DQ8 in IBD would explain a similar or even reduced CeD expression in IBD.

Some studies have looked for a relationship between HLA class Ⅱ molecules and IBD. Most studies have

analyzed HLA alleles instead of complete haplotypes. They observed a tendency to lower frequency of HLA-DQ2 or -DQ8[17,18], and, mainly, -DR3 and -DR4 (because most studies are from the serologic HLA era), in IBD, as well as a higher frequency of HLA-DR7[17]. A study of DiGiacomo *et al*[19], which analyzed complete haplotypes in several immune-related diseases, found a reduced frequency of HLA-DQ2 and -DQ8 in IBD, although only 36 IBD patients were included.

The main objective of the study was to determine whether or not IBD patients are genetically predisposed to CeD; we conducted a study to analyze the frequency of CeD-related HLA (alleles encoding DQ2 and DQ8 dimers: HLA-CeD) in our IBD population [both in patients with ulcerative colitis (UC) as with Crohn's disease (CD)]. An analysis of HLA-CeD frequencies according to sex was also performed in our IBD population.

MATERIALS AND METHODS

Patients and controls

The study included 1034 subjects from the Community of Valencia, Spain: 457 adult patients with IBD and 577 organ donors HLA-typed at the Transfusion Center of the Valencian Community (TCVC). IBD patients cared from the out-patient-clinic and the IBD day-care unit of the University Clinic Hospital of Valencia, were prospectively and consecutively retrieved.

Clinical information was updated and gathered from the patients, the physical and online record and by means of our clinical database. Ethnicity, age, sex, diagnosis (CD or UC), disease location (Montreal Classification and anastomosis)[20], extraintestinal manifestations (arthralgia, ankylosing spondylitis, sacroiliitis, aphthous stomatitis, dermatologic, ocular and thrombotic events, and primary sclerosing cholangitis), disease complications (megacolon, hemorrhage, perforation and intraabdominal abscesses) and need of surgery were all recorded.

The blood was analyzed at the Histocompatibility Department of the TCVC (EFI Accreditation number: 09-ES-014.986) to determine the presence or absence of CeD risk HLA-haplotypes: haplotype HLA-DQA1*05:01-DQB1*02:01 (HLA-DQ2.5*cis*), the heterozygotic genotype HLA-DQA1*02:01-DQB1*02:02 + DQA1*05:05-DQB1*03:01 (HLA DQ2.2 + HLA-DQ7.5, HLA-DQ2.5*trans*), and haplotype HLA-DQA1*03-DQB1*03:02 (HLA-DQ8). HLA was considered to predispose for CeD (HLA-CeD) when one of these haplotypes was present.

Approval from the hospital's Ethics Committee was obtained (Ethics Committee record nº 238), as well as written informed consent from each participating subject.

HLA genotyping

HLA-DQA1 and -DQB1 low- and high-resolution genotyping was performed by polymerase chain reaction with sequence-specific primers (PCR-SSP) according to the method described by Olerup *et al*[21]. Genomic DNA was isolated from nucleated cells by Magtration® technology[22]. Each PCR reaction was performed on about 80 ng of extracted DNA, using 0.15 units of Taq DNA polymerase (AmpliTaq® DNA Polymerase, Applied Biosystems, The Netherlands), and Olerup commercial primers (Olerup SSP AB, Stockholm, Sweden), according to the manufacturer's instructions. PCR was carried out in a final volume of 10 μL in a GeneAmp PCR System 9700 (Applied Biosystems, The Netherlands). An initial denaturizing step at 94 ℃ for 2 min was followed by 10 two-temperature cycles (94 ℃ for 10 s and 65 ℃ for 60 s) and 20 three-temperature cycles (94 ℃ for 10 s, 61 ℃ for 50 s and 72 ℃ for 30 s). Detection of amplified alleles was carried out by agarose gel electrophoresis.

Statistical analysis

According to the published data, the prevalence of HLA-DQ2 and DQ8 in the Spanish general population is about 30%[8]. Therefore, considering a 95% confidence level (type I error of 0.05, and 0.8 statistical power), we needed a sample size of 323 patients to detect significant differences between groups.

Statistical analysis was done using the PASW 17.0 software (SPSS Inc, Chicago, IL, United States), Microsoft Office Excel 2003 and STATGRAPHICS Plus (version 5.1). We calculated the absolute and relative frequencies of the different variables. We considered significant a P value of less than 0.05. Statistical methods used were: χ^2 test, logistic regression to calculate the odds ratio (OR), and ANOVA tests. The attributable risk was measured using the phenotypic frequency, relative risk (RR), etiologic fraction -EF: risk that genes can confer for the development of a disease- (for RR > 1) and preventive fraction -PF: protection that genes confer- (for RR < 1).

The Holm-Bonferroni correction was used to determine if the relationship between an allele or a group of alleles with a disease (or phenotype expression) was true, when multiple determinations were made.

RESULTS

The study included 1034 caucasian subjects from the Community of Valencia, Spain. 457 adult patients with IBD (202 females and 255 males) were retrieved from January 2007 to March 2011. All patients had been diagnosed with IBD according to accepted clinical, endoscopic, radiological, and histological findings[23,24]. 250 patients had CD and 207 UC. The phenotypical characteristics of the IBD patients are listed in Table 1. The control group was made up of 577 (220 females and 357 males) unrelated organ donors HLA-typed at the Transfusion Center of the Valencian Community

Table 1 Phenotypical characteristics of inflammatory bowel disease patients *n* (%)

	All IBD patients	Crohn's disease patients	UC patients
Number of patients	457 (100)	250 (55)	207 (45)
Gender (female)	202 (44)	122 (49)	80 (39)
Disease location		L1: 60 (24.0), L2: 30 (12.0), L3: 128 (51.2), L1 + L4: 11 (4.4), L2 + L4: 1 (0.4), L3 + L4: 20 (8.0), perianal: 93 (37.2)	Proctitis: 19 (9.2), left colitis: 60 (29.0), extensive colitis: 128 (61.8)
Disease behavior		B1: 88 (35.2), B2: 102 (40.8), B3: 60 (24.0)	
Complications	54 (12)	46 (19.7)	8 (4.1)
Extraintestinal manifestations	163 (35.7)	100 (41.8)	63 (31.7)
Surgery[1]	166 (37.5)	146 (59.3)	20 (10.2)

[1]IBD related surgery: Intestinal or perianal; L1: Distal ileum; L2: Colonic; L3: Ileocolonic; L4: Upper disease; B1: Inflammatory (nonstricturing/nonpenetrating); B2: Stenotic/stricturing and B3: Penetrating/fistulizing.

Table 2 HLA-CeD, -DQ2.5 and -DQ8 for all inflammatory bowel disease patients, ulcerative colitis patients, and Crohn's disease patients, compared with controls

HLA	Controls (*n* = 577)	IBD (*n* = 457)	Crohn's disease (*n* = 250)	Ulcerative colitis (*n* = 207)
HLA-CeD	227, 39.34% (95%CI: 35.36%-43.33%)	156, 34.14% (95%CI: 29.78%-38.48%), *P* = 0.0852	90, 36% (95%CI: 30.05%-41.95%), *P* = 0.364	66, 31.88% (95%CI: 25.54%-38.23%), *P* = 0.0571, PF = 11%
HLA-DQ2.5	137, 23.74% (95%CI: 20.27%-27.22%)	99, 21.66% (95%CI: 17.89%-25.44%), *P* = 0.381	65, 26.0% (95%CI: 20.6%-31.4%), *P* = 0.4879	34, 16.43% (95%CI: 11.38%-21.47%), *P* = 0.0287, PF = 8%
HLA-DQ8	101, 17.50% (95%CI: 14.40%-20.60%)	60, 13.13% (95%CI: 10.03%-16.23%), *P* = 0.054, PF = 5%	28, 11.20% (95%CI: 7.29%-15.11%), *P* = 0.0217, PF = 7%	32, 15.46% (95%CI: 10.53%-20.38%), *P* = 0.501
HLA-DQ2.2	131, 22.70% (95%CI: 19.29%-26.12%)	134, 29.32% (95%CI: 25.15%-33.50%), *P* = 0.022, EF = 9%	85, 34% (95%CI: 28.13%-39.88%), *P* = 0.001, EF = 15%	49, 23.67% (95%CI: 17.88%-29.46%), *P* = 0.856

HLA: Human leucocyte antigen; PF: Preventive fraction.

(TCVC).

HLA-CeD was found in 37.0% (383 subjects) of the study population**.** HLA-CeD was more frequent in the control group: 39.34% *vs* 34.14% in IBD patients, but this difference did not reach statistical significance (Table 2).

HLA-CeD was found in 31.88% of the UC patients and 36% of the CD subjects (Table 2). We compared the frequencies of HLA-CeD in controls *vs* CD and UC and observed a tendency to a lower frequency of HLA-CeD in UC patients *vs* controls (*P* = 0.0571), with a preventive fraction (PF) of 11% (HLA-CeD could confer 11% protection from developing UC) and no differences between CD patients and controls.

Women with IBD had a lower frequency of HLA-CeD than the control women (34% *vs* 43%, *P* = 0.0565; OR = 0.68 and PF = 14%) According to the type of IBD we observed that UC female patients had HLA-CeD less frequently than controls, although it did not reach significance (*P* = 0.0613).

These tendencies became significant when exploring the frequencies of the different HLA-CeD haplotypes, by gender and type of IBD. HLA-DQ2 was less frequent in UC patients and HLA-DQ8 in CD patients. The frequency of HLA-DQ2.5 in UC patients (16.43%) was significantly lower than the one of the control group (23.74%), with a PF of 8% (Table 2). This was observed when calculating frequencies of HLA-DQ2.5*cis* only, *trans* alone and global frequencies (both *cis* and *trans*). In considering both sexes together, the presence of DQ2.5cis was significantly lower in UC, with a frequency of DQ2.5cis of 20.28% in controls compared to 13.53% in patients with UC (*P* = 0.0319; PF = 7%). When taking into account only UC women *vs* control women (16.25% of HLA-DQ2.5 *vs* 27.44%), the probability obtained with a logistic regression model of developing UC in women with HLA-DQ2.5 was reduced almost 50% (*P* = 0.0466), with a PF of 13%. In women with UC, the frequency of DQ2.5cis was reduced more than 50%, given that it was multiplied by 0.459 (*P* = 0.0344).

HLA-DQ8 also showed a tendency to be less frequent in IBD patients (13.13%) than controls (17.50%), mainly due to the significantly reduced frequency of -DQ8 in CD patients (11.20%) (Table 2). No differences between genders were seen.

HLA-DQ2.2 was significantly more frequent in CD patients (34%) than in controls (22.7%) or than in UC patients (23.67%), (Table 2). Of the patients with CD, 31% of the males and 37% of the females have HLA-DQ2.2, while only 26% and 24% of males with UC and controls, respectively, and 20% and 21% of females with UC and controls, respectively, have it. No statistically significant differences between sexes were seen.

DISCUSSION

Some authors consider CD[25] or UC[26] patients at high risk of presenting CeD[27-32] and others don't[26,32,33]. However, none determine the frequency of HLA-CeD in their patients, although Leeds points out that

HLA-CeD could be less frequent in IBD patients than in the general population[33]. A more recent article determines HLA-CeD frequency in functional and organic gastrointestinal diseases. They observe that HLA-CeD is not more frequent in the IBD group than in the controls, but the sample size of IBD patients (36 IBD patients) is very small[19].

Genome studies have observed that CeD and IBD share some non-*HLA* gene[33-36]. Analyzing the published studies of HLA-alleles related to IBD[18], we could deduce that the HLA-CeD alleles are less frequent in IBD (DR4, usually linked to DQ8, in UC, and DR3, normally linked to DQ2, in CD), but there are no published prevalences of them. This brings us to the possible conclusion that, in IBD and CeD, there is an overlap of non-HLA genes but maybe not of HLA-CeD genes, and it poses the question of if this could explain why CeD is not more frequent in IBD.

We performed this study to determine if the IBD population is an "at-risk" group for CeD by determining the genetic predisposition for this disease in IBD patients. We aimed to quantify the frequency of celiac disease-related HLA (HLA-DQ2 and HLA-DQ8) in the IBD population, compare it to that of the general population, and observe if it was related to a specific IBD phenotype or gender.

With the benefit (to draw conclusions, genetic studies need homogeneous populations) and the limitation of such a homogeneous study group, the results must be interpreted taking into account the 100% Caucasian race of both cases and controls.

In our study no statistical differences in HLA-CeD frequency were detected between the IBD group and the control group. DiGiacomo *et al*[19] analyze HLA-DQ2 and -DQ8 in several digestive diseases, including IBD. Their results are similar to ours, but they cannot be extrapolated because only 36 IBD patients are analyzed[19]. They obtain a prevalence of HLA-CeD in IBD patients of 38.9%, with no differences when compared to a previously published HLA-CeD frequency in the general Italian population (39%).

In addition to DiGiacomo's article, we have found no studies that analyze the complete heterodimers DQα + DQβ related to CeD, *i.e.*, not only alleles, nor their actual frequency in IBD patients. Most of the studies do not take into account gender, which is important in CeD because of the 2:1 preponderance of females. We observed that women with IBD tend to show differences with women in the control group, with a preventive fraction of 14%.

Although both CeD and IBD have chronic intestinal inflammation, with increased intestinal permeability and an altered immune response, the different genetic basis is probably responsible for the different interaction with the environment. This is better understood when exploring the individual HLA-DQ2.5 and -DQ8 haplotypes, which might even confer protection from the future development of IBD, as our results show.

Taking into account the type of IBD, we can see that HLA-CeD tends to be less frequent in UC patients (32%) than in CD (36%) and controls (39%), confering 11% protection from developing UC. These differences are even more notable when analyzing only women: UC 31%, CD 36% and 43% controls. The lower percentages of HLA-CeD in women with IBD are mainly justified by the lower prevalence of the heterodimer HLA-DQ2.5cis, which confers a preventive fraction of 9%.

To delve more deeply into the tendency of HLA-CeD being a protection factor against IBD, the interaction of the variables sex, type of IBD and frequency of HLA-CeD was analyzed for statistical significance. We observed that the frequency of DQ2.5cis was reduced more than 50%, in women with UC. The reduction in the risk of HLA-DQ2.5cis in UC was not only observed in women (PF: 13%); in considering both sexes together, the presence of DQ2.5cis was significantly lower in UC (PF = 7%). Similar results were obtained when analyzing both *cis* and *trans* HLA-DQ2.5 together: HLA-DQ2.5 was significantly reduced in the UC group, and even more remarkably in UC women. This demonstrated that being a woman with HLA-DQ2.5 bears 13% protection from developing UC.

According to our results, HLA-DQ8 is also less frequent in IBD patients than those in the control group. HLA-DQ8 was significantly reduced in CD patients (PF = 7%).

Summarizing, the frequency of HLA-CeD in IBD patients is similar to the general population; however, there is a significant decrease in the number of UC patients with HLA-DQ2.5 and of Crohn's disease patients with HLA-DQ8. The preventive fractions that oscillate between 5% and 14% suggest that CeD haplotypes protect from developing IBD. More specifically, HLA-DQ2.5 guards against the appearance of UC and HLA-DQ8 against the initiation of CD. The low preventive fractions are explained by the fact that CD and UC are multifactor illnesses that include many phenotypes, in which, save exceptions such as families with specific altered genes like IL-10, various factors interact to produce the disease.

Since HLA-DQ2.5 is the most closely related to CeD, and the most frequent in CeD, our results may suggest that the risk of celiac disease is lower in patients with IBD and, therefore, its expression as well. Extensive studies with duodenal biopsies from Spanish patients should be carried out to see if, as in the Italian study by Casella *et al*[37], the prevalence of CeD in IBD is lower than in the general population.

The role of the HLA-DQ 2.2 dimer (DQA1*02:01 + DQB1*02:02) is controversial in terms of its contribution to the predisposition to celiac disease. The majority of authors are detractors of the role of predisposition to CeD of HLA - DQ2.2[38]. One even suggests that it may act as a protective factor[39], but there is also an author who notes that it clearly predisposes to CeD[40]. In the

meta-analysis by Stokkers *et al*[18] a positive association of HLA-DRB1*07 was observed in CD patients, a gene that is normally closely bound to HLA-DQ2.2. The Italian study by Lombardi *et al*[41] in 2001 also observed that the haplotype DRB1*07-DQB1*02:02 was the most frequent in their population. The frequency of HLA-DR7 in the European CD population is high[42-44], ranging between 5% and 29%, unlike the Japanese, where it is only found in 1%[44].

Our study observed that the frequency of HLA-DQ2.2 was greatly increased in patients with CD; more than a third of the patients carry this haplotype. The relative risk of CD in patients with HLA-DQ2.2 is 1.75 (EF = 15%). Thus, the contribution of HLA-DQ2.2 as a risk factor of CD development is 15%.

As in other European studies, such as the Spanish study by Fernandez *et al*[45], where the HLA-DRB1*07 is found in a high proportion of CD patients with ileal involvement, in our population with IBD there is a high frequency of HLA-DQ2.2 among patients with CD with ileal involvement (35.5% of patients with ileal Crohn have HLA-DQ2.2). In a large-scale, international genetics study, published in 2016, Cleynen *et al*[46] observed a strong relationship between HLA-DRB1*07 and Crohn's disease.

This suggests that HLA-DQ 2.2 may be a supplementary tool to diagnose undetermined IBD. Future studies have to be performed to evaluate if using HLA-DQ2.2 can help reach a diagnosis or if it can be of use for IBD family-members' follow-up.

Is the IBD population an "at-risk" group for celiac disease? According to our results, genetically no. They have the same frequency of CeD-related HLA haplotypes globally and even a lower frequency of them when specifically looking at UC or CD, and gender.

In conclusion, our results, not only quantify the frequency of celiac disease related HLA haplotypes in the IBD population, but also show that they are not more frequent in the IBD population, and even more, that HLA-DQ2 is less frequent in UC patients, especially women, and HLA-DQ8 in CD patients, and that these haplotypes confer low grade protection from the development of future IBD. Our results also confirm a high frequency of HLA-DQ2.2 in our CD patients, and point out that HLA-DQ2.2 may actually act as a genetic risk factor for a future diagnosis of CD.

ARTICLE HIGHLIGHTS

Research background

Celiac disease (CeD) and inflammatory bowel disease (IBD) are chronic intestinal disorders with progressively increasing incidences and prevalences. Both diseases are thought to be secondary to the interaction of certain environmental factors which either directly cause or enable others to trigger the disease (gluten -cause of CeD-, infections, dysbiosis, *etc.*), in genetically predisposed patients, by producing an altered immunological response. CeD has defined diagnostic criteria, which include blood antibodies, genetic testing, upper endoscopy findings and, especially, histological small-bowel changes. The involvement of human leucocyte antigen (HLA) genes codifying HLA-DQ2 and -DQ8 antigens in the susceptibility to the disease is clearly established and HLA typing has been accepted as a useful test to exclude CeD, because only 0.5 % of CeD patients lack both DQ2 and DQ8 antigens. IBD patients have historically been considered to be at higher risk for CeD, which could be supported by the fact that IBD and CeD are quite prevalent and due to a theoretically similar pathogenesis, with the interaction of genetic, immunological, and environmental factors (gut flora, gastroenteritis, *etc.*). Two more recent studies have analyzed CeD-related antibodies and biopsies and observed that CeD is just as frequent or even less in the IBD population, but CeD is still included as a more prevalent disease in IBD in some texts. None of them have analyzed the frequency of HLA-DQ2 and 8 (HLA-CeD) in IBD patients. Only one study has done so but it only included 36 patients.

Research motivation

We wanted to know if IBD patients are genetically predisposed to CeD. Since negative HLA-CeD has a very high predictive negative value, not having it discards having CeD in most cases. We wanted to determine the frequency of HLA-CeD in IBD, which has never been calculated, and whether having the haplotypes is related to having ulcerative colitis (UC) or Crohn's disease (CD).

Research objectives

To determine whether or not IBD patients are genetically predisposed to CeD, we conducted a study to determine the frequency of CeD-related HLA (alleles encoding DQ2 and DQ8 dimers: HLA-CeD) in our IBD population (both in patients with UC as with CD). An analysis of HLA-CeD frequencies according to sex was also performed in our IBD population.

Research methods

We conducted a prospective study with IBD patients from our Unit. Clinical information was gathered and blood was tested for HLA-CeD. The control group was made up of unrelated Valencian organ donors.

Research results

A total of 1034 patients were analyzed: 457 IBD (207 UC, and 250 CD) patients and 577 healthy controls. 39% of the controls and 34% of the patients had HLA-CeD (P = 0.0852). HLA-DQ2 was less frequent in UC patients (P = 0.0287), and HLA-DQ8 in CD (P = 0.0217). In women with UC, the frequency of DQ2.5cis (DQB1*02:01-DQA1*05:01) was reduced $\geqslant$ 50% [P = 0.0344; preventive fraction (PF) = 13%]. PFs (7%-14%) were obtained with all HLA-CeD haplotypes. HLA DQB1*02:02-DQA1*02:01 (HLA-DQ2.2) was more frequent in CD patients with respect to controls (P = 0.001) and UC patients (etiological fraction = 15%).

Research conclusions

HLA-CeD is not more frequent in IBD patients, with an even lower frequency of HLA-DQ2 and -DQ8 in UC and CD respectively. HLA-DQ2.5 confers protection from the development of UC, especially in women, and HLA-DQ8 does so for the appearance of CD. HLA-DQ2.2 is present in 34% of the CeD patients and may constitute a genetic risk factor for CeD development. This helps answer the ongoing question of whether or not IBD patients have a higher risk of CeD. According to our study, IBD patients have the same genetic predisposition of CeD than the general population, showing an even lower frequency when subanalyzing by haplotypes and type of IBD. To our knowledge, it is the first time a frequency of the HLA-CeD haplotypes is given in a large enough IBD population. We also found a high frequency of HLA-DQ2.2 in Crohn's disease, pointing to it as a risk factor.

Research perspectives

This study supports the change in trend of the relationship between CeD and IBD, confirming it is not more frequent. We found HLA-DQ2 was less frequent in UC and HLA-DQ8 in CD, but we did not find any relationship with the presence or absence of CeD haplotypes and certain IBD phenotypes. We might need larger studies to find if these alleles can be related to phenotypes. Future studies will help confirm HLA-DQ 2.2 as a risk factor for Crohn's. This could help decision taking in unclear cases (example with indeterminate colitis). Studies are also needed to see if to correlates with disease severity.

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ORIGINAL ARTICLE

Observational Study

Surgical specimen extraction *via* a prophylactic ileostomy procedure: A minimally invasive technique for laparoscopic rectal cancer surgery

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Abstract

AIM

To retrospectively evaluate the safety and feasibility of surgical specimen extraction *via* a prophylactic ileostomy procedure in patient with rectal cancer.

METHODS

We systematically reviewed 331 consecutive patients who underwent laparoscopic anterior resection for rectal cancer and prophylactic ileostomy in our institution from June 2010 to October 2016, including 155 patients who underwent specimen extraction *via* a prophylactic ileostomy procedure (experimental group), and 176 patients who underwent specimen extraction *via* a small lower abdominal incision (control group). Clinical data were collected from both groups and

statistically analyzed.

RESULTS

The two groups were matched in clinical characteristics and pathological outcomes. However, mean operative time was significantly shorter in the experimental group compared to the control group (161.3 ± 21.5 min *vs* 168.8 ± 20.5 min; P = 0.001). Mean estimated blood loss was significantly less in the experimental group (77.4 ± 30.7 mL *vs* 85.9 ± 35.5 mL; P = 0.020). The pain reported by patients during the first two days after surgery was significantly less in the experimental group than in the control group. No wound infections occurred in the experimental group, but 4.0% of the controls developed wound infections (P = 0.016). The estimated 5-year disease-free survival and overall survival rate were similar between the two groups.

CONCLUSION

Surgical specimen extraction *via* a prophylactic ileostomy procedure represents a secure and feasible approach to laparoscopic rectal cancer surgery, and embodies the principle of minimally invasive surgery.

Key words: Minimally invasive surgery; Rectal cancer; Anastomotic leakage; Prophylactic ileostomy; Safety

Core tip: Prophylactic ileostomy plays an important role in reducing the incidence of anastomotic leakage in rectal cancer patients. In this paper, we introduce an innovative method named surgical specimen extraction *via* a prophylactic ileostomy procedure and to evaluate its safety and feasibility.

Wang P, Liang JW, Zhou HT, Wang Z, Zhou ZX. Surgical specimen extraction *via* a prophylactic ileostomy procedure: A minimally invasive technique for laparoscopic rectal cancer surgery. *World J Gastroenterol* 2018; 24(1): 104-111 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i1/104.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i1.104

INTRODUCTION

Rectal cancer patients with ultra-low anastomosis (anastomosis level ⩽ 5 cm from the anal verge) have a high incidence of anastomotic leakage (AL), with the reported rates ranging from 9.4% to 12.3%[1-3]. In addition, several studies have shown that male sex (P < 0.001), body mass index (BMI) ⩾ 25 kg/m^2 (P = 0.05), American Society of Anesthesiologists (ASA) score ⩾ 3 (P = 0.04), tumor size ⩾ 5 cm (P = 0.05), preoperative chemoradiotherapy history (P = 0.02), long operative time (P = 0.0002), and number of stapler firings ⩾ 3 (P < 0.001) are all risk factors for AL in rectal cancer patients[4-10]. AL is a serious complication in that it extends hospitalization time, increases expense, delays subsequent therapy, and increases perioperative mortality[5-7].

Studies show that prophylactic ileostomy plays an important role in reducing the incidence of AL in patients with one or more of the above-mentioned factors[11-14]. During conventional laparoscopic anterior resection of rectal cancer, a vertical or horizontal incision in the lower abdomen about 5 cm long was utilized to extract the specimen, and then a circular incision about 4 cm in diameter was made in the right lower quadrant to complete the ileostomy. With ongoing developments in minimally invasive surgery, we tried an innovative method at the National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College beginning in June 2010 that involved surgical specimen extraction through a prophylactic ileostomy procedure so as to avoid making a vertical or horizontal incision in the lower abdomen. Herein, we describe this surgical innovation and analyze its safety and feasibility.

MATERIALS AND METHODS

Patients and clinical protocol

The ethics committee at our institution approved this retrospective study, and it conformed to the ethical standards of the World Medical Association Declaration of Helsinki. The data from all consecutive patients with rectal cancer who underwent laparoscopic anterior resection and prophylactic ileostomy in our institution from June 2010 to October 2016 were compiled and included in this study. All of these patients had been diagnosed with rectal cancer before undergoing surgery. Preoperative examinations, including routine blood tests, serum carcinoembryonic antigen (CEA), chest radiography, electrocardiograms, abdominal and pelvic computed tomography scans, and pelvic magnetic resonance imaging, were used to evaluate the operative approach. The patients themselves selected the surgical procedure after the benefits and risks were explained explicitly. All of the surgeons in this study performed both types of surgery. The pathological specimens were examined by two pathologists who specialized in colorectal cancer.

Tumor staging was performed based on the criteria from the 7th edition of the American Joint Committee on Cancer (AJCC) manual. Postoperative pain was evaluated by the patients using the "visual analog scale" (VAS) of 0 to 10, with 0 representing no pain and 10 representing the worst pain imaginable. Clinical characteristics, operative outcomes, pathological outcomes, postoperative complications, and follow-up information were recorded in our database.

Surgical procedure

In the operating room, anesthesia was induced, and

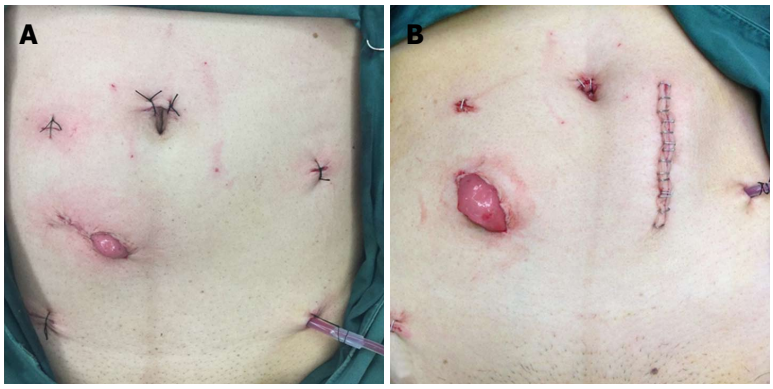

Figure 1 Specimen extraction *via* a prophylactic ileostomy procedure or an incision. A: Specimen extraction *via* a prophylactic ileostomy procedure. After cutting off the distal intestine, the surgical specimen was extracted from the abdominal cavity *via* the stoma protected by the single-use incision protector. And then the ileostomy procedure was performed after finishing the anastomosis. B: Specimen extraction *via* a small lower abdominal incision. After cutting off the distal intestine, the surgical specimen was extracted from the abdominal cavity *via* the vertical incision protected by the single-use incision protector. And then the ileostomy procedure was performed after finishing the anastomosis.

the patient was placed in the modified lithotomy position for the laparoscopic procedure. A four or five-port technique was used. The surgeon and the camera operator stood on the right of the patient, while the assisting surgeon stood on the left. Abdominal inflation pressure was maintained at approximately 15 mmHg. Division of blood vessels, dissection of lymph nodes, and abscission of the distal intestine were then performed laparoscopically. Total mesorectal excision (TME) principles were followed.

In the experimental group, the specimen extraction was performed *via* an incision with a diameter of about 4.5 cm in the right lower quadrant, and then the surgical specimen was extracted. After abscising the proximal intestine and implanting the stapling head, the pneumoperitoneum was re-established, and a colorectal anastomosis was performed using a stapling technique. Finally, the ileostomy procedure was performed *via* the incision (Figure 1A). In the control group, a vertical or horizontal incision with a length of about 5 cm was made in the lower abdomen to extract the specimen, and an anastomosis was done by the same method as in the experimental group, and then a circular incision with a diameter of about 4 cm was performed in the right lower quadrant to complete the ileostomy procedure (Figure 1B).

The single-use incision protectors were used to protect incisions from pollution or cancer cell implantation for both procedures when taking out the surgical specimen *via* the incision. Laparoscopic surgery had been planned for all of the patients, but it was necessary to convert several of the procedures to open surgery.

Follow-up

After hospital discharge, patients were advised to have follow-up monitoring by their doctors every 3 mo during the first 2 years, every 6 mo for the next 3 years, and then yearly visits after 5 years. The beginning of the follow-up time was set at the first day after surgery, and the end was set at June 30, 2017.

Definitions

The positive circumferential resection margin (CRM) was considered as microscopic tumor less than 1 mm from the mesorectal fascia. Disease free survival (DFS) was defined as the period from surgery to death or disease recurrence. Overall survival (OS) was defined as the period from surgery to death.

Statistical analysis

Patients who required conversion were included in their intended group, because the data were analyzed on an intention-to-treat basis. Quantitative data that were normally distributed are presented as mean ± SD, and were analyzed by the Student's *t*-test. Categorical data are presented as number and percentage, and were analyzed by the chi-squared test or Fisher's exact test. Survival analysis was performed by the Kaplan-Meier method, and survival was compared by the log-rank test. All the tests were two-sided, with a *P*-value < 0.05 used as the threshold for statistical significance. The Statistical Package for the Social Sciences (SPSS) version 21.0 for Windows (IBM Corp, Armonk, NY, United States) was used for data analyses.

RESULTS

Clinical characteristics

A total of 331 consecutive cases were included in our study, including 155 (46.8%) cases in the experimental group and 176 (53.2%) in the control group. There were no significant differences in terms of gender, age, BMI, ASA score, or history of neoadjuvant chemoradiation between two groups (Table 1). No deaths occurred during the perioperative period.

Operative outcomes

Operative outcomes are shown in Table 2. Mean opera-

Table 1 Clinical characteristics *n* (%)

Characteristic	Experimental group (*n* = 155)	Control group (*n* = 176)	*P* value
Gender			0.842
Male	88 (56.8)	98 (55.7)	
Female	67 (43.2)	78 (44.3)	
Age (yr; mean ± SD)	55.5 ± 12.2	57.0 ± 11.6	0.250
BMI (kg/m^2, mean ± SD)	23.9 ± 2.5	24.4 ± 3.1	0.171
ASA score			
1	40 (25.8)	47 (26.7)	0.857
2	79 (51.0)	82 (46.6)	
3	31 (20.0)	41 (23.3)	
4	5 (3.2)	6 (3.4)	
Neoadjuvant chemoradiation			
Yes	48 (31.0)	52 (29.5)	0.811
No	107 (69.0)	124 (70.5)	

BMI: Body mass index; ASA: American Society of Anesthesiologists.

Table 2 Operative outcomes

Variable	Experimental group (*n* = 155)	Control group (*n* = 176)	*P* value
Operating time (min; mean ± SD)	161.3 ± 21.5	168.8 ± 20.5	0.001
Estimated blood loss (mL; mean ± SD)	77.4 ± 30.7	85.9 ± 35.5	0.020
Diameter of stoma (cm; mean ± SD)	4.7 ± 0.5	4.0 ± 0.6	< 0.001
Time to first flatus (d; mean ± SD)	1.2 ± 0.4	1.3 ± 0.3	0.586
Time to first oral intake (d; mean ± SD)	1.9 ± 0.4	2.0 ± 0.5	0.062
Postoperative hospitalization (d; mean ± SD)	6.3 ± 1.3	6.5 ± 1.2	0.199
Postoperative pain score (mean ± SD)			
The first day	2.6 ± 0.8	3.1 ± 1.1	< 0.001
The second day	1.6 ± 0.6	1.8 ± 0.7	0.012
The third day	0.6 ± 0.5	0.6 ± 0.6	0.628
No. of conversions to open surgery (%)	5 (3.2)	6 (3.4)	1.000

tive time was significantly shorter in the experimental group compared with the control group (161.3 ± 21.5 min *vs* 168.8 ± 20.5 min, $P = 0.001$). Mean estimated blood loss was significantly less in the experimental group than in the control group (77.4 ± 30.7 mL *vs* 85.9 ± 35.5 mL, $P = 0.020$). The mean diameter of the stoma was significantly larger in the experimental group (4.7 ± 0.5 cm *vs* 4.0 ± 0.6 cm, $P < 0.001$). The pain on the VAS reported by patients in the experimental group during the first two days after surgery was significantly reduced compared to the control group ($P < 0.001$ and $P = 0.012$, respectively). There were no significant differences in time to first flatus, time to first oral intake, or postoperative hospitalization. There were five and six cases of conversion to open surgery in the experimental group and the control group, respectively ($P = 1.000$).

Pathological outcomes

Pathological results are summarized in Table 3. There were no significant differences in the findings, including tumor size, lengths of the proximal and distal resection margins, number of lymph nodes harvested, and number of patients with < 12 lymph nodes harvested, differentiation degree, pathological patterns, nerve and vessel invasion, or pTNM stage. There were no cases with positive distal margins or positive CRM in either group.

Postoperative complications

Postoperative complications are summarized in Table 4. Although the average diameter of the stoma was larger in the experimental group, there were no statistically significant differences in the incidence of stoma-related complications, including infections, retractions, bleeding, or parastomal hernia. The incidence of wound infections was lower in the experimental group than in the control group (0% *vs* 4.0%, $P = 0.016$). Three patients underwent reestablishment of stoma because of stoma necrosis, and five patients underwent debridement and suturing procedure because of incision infection in the control group. No statistical differences were found with respect to intestinal obstruction, urinary retention, cardiopulmonary complications, or re-operation.

Survival outcomes

The mean follow-up period was 40 mo (range 7-84 mo) in the experimental group and 41 mo (range 6-84 mo) in the control group ($P = 0.781$). No patients suffered from local recurrence in the specimen extraction sites in both groups. The estimated 5-year DFS rate was 60.0% in the experimental group and 64.5% in the

Table 3 Pathological outcomes *n* (%)

Variable	Experimental group (*n* = 155)	Control group (*n* = 176)	*P* value
Tumor size (cm; mean ± SD)	4.0 ± 0.8	4.1 ± 0.8	0.205
Proximal resection margin (cm; mean ± SD)	16.2 ± 7.1	15.4 ± 7.3	0.309
Distal resection margin (cm; mean ± SD)	2.2 ± 0.4	2.2 ± 0.4	0.477
No. of lymph nodes harvested (mean ± SD)	22.8 ± 5.3	21.7 ± 5.0	0.375
No. of patients with lymph nodes harvested < 12	10 (6.5)	13 (7.4)	0.830
Differentiation degree			0.963
Good	45 (29.0)	50 (28.4)	
Moderate	62 (40.0)	73 (41.5)	
Poor	48 (31.0)	53 (30.1)	
Pathological pattern			0.785
Canalicular adenocarcinoma	108 (69.7)	125 (71.0)	
Mucinous adenocarcinoma	25 (16.1)	22 (12.5)	
Papillary adenocarcinoma	12 (7.7)	16 (9.1)	
Signet-ring carcinoma	10 (6.5)	13 (7.4)	
Nerve invasion	30 (19.4)	37 (21.0)	0.706
Vessel invasion	26 (16.8)	30 (17.0)	0.948
Positive distal margin	0 (0)	0 (0)	N/A
Positive CRM	0 (0)	0 (0)	N/A
pTNM stage			0.807
Ⅰ	38 (24.5)	45 (25.6)	
Ⅱ	61 (39.4)	70 (39.8)	
Ⅲ	44 (28.4)	52 (29.5)	
Ⅳ	12 (7.7)	9 (5.1)	

SD: Standard deviation; CRM: Circumferential resection margin; pTNM: Pathological tumor-node-metastasis; N/A: Not applicable.

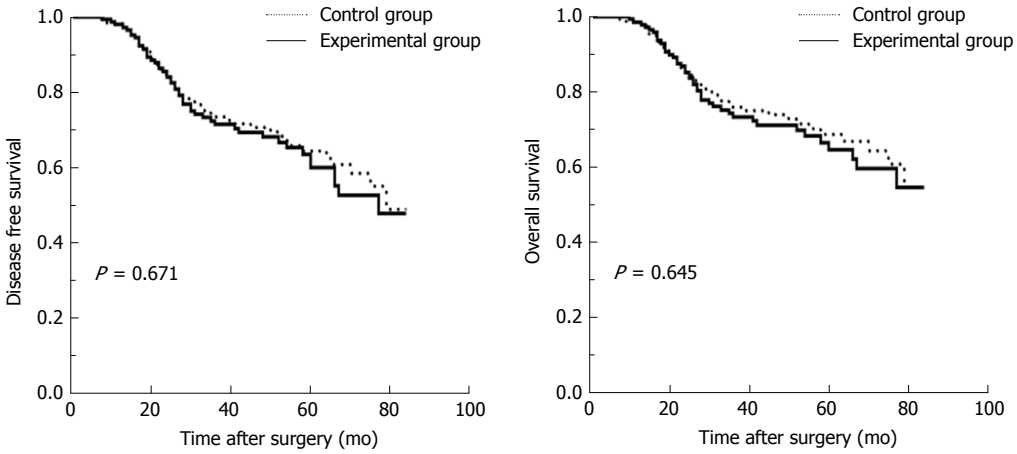

Figure 2 Disease free survival and overall survival of patients in the experimental and control groups.

control group (P = 0.671). The estimated 5-year OS rate was 64.8% in the experimental group and 68.8% in the control group (P = 0.645) (Figure 2).

DISCUSSION

With increased life expectancy and improved standards of living, the incidence of rectal cancer is increasing in China[15]. The most distinguishing feature of rectal cancer in the Chinese population is that the majority (60%-75%) are lower rectal cancer, which has higher proportions than those reported in Western populations[16,17]. Surgery is the mainstay of treatment for rectal cancer. In the past, abdominoperineal resection was the standard of care, with sigmoidostomy negatively impacting the patients' quality of life. At present, the exciting prospect is that there is an increased probability of successfully performing re-anastomosis and anal-sparing procedures for patients with lower rectal cancer as a result of the advances in surgical technology and improvements in operative procedures[18,19].

However, the possibility of AL is greater for cases with ultra-low anastomoses, which can increase the perioperative mortality and affect long-term survival. There are not only procedure-related factors, such as longer operative time, ⩾ 3 stapler firings, and ultra-low anastomosis, but also demographic risk factors, such as male sex, BMI ⩾ 25 kg/m^2, ASA score ⩾ 3, tumor size ⩾ 5 cm, or a history of preoperative

Table 4 Postoperative complications *n* (%)

Variable	Experimental group (*n* = 155)	Control group (*n* = 176)	*P* value
Stoma related			
Stoma edema	15 (9.7)	18 (10.2)	0.868
Stoma infection	4 (2.6)	2 (1.1)	0.326
Stoma retraction	1 (0.6)	0 (0)	0.468
Stoma necrosis	0 (0)	3 (1.7)	0.251
Stoma fistula	0 (0)	1 (0.6)	1.000
Stoma bleeding	1 (0.6)	0 (0)	0.468
Stoma stenosis	0 (0)	2 (1.1)	0.501
Skin inflammation around the stoma	16 (10.3)	16 (9.1)	0.705
Parastomal hernia	4 (2.6)	2 (1.1)	0.424
Mucosal prolapse	3 (1.9)	2 (1.1)	0.668
Wound infection	0 (0)	7 (4.0)	0.016
Intestinal obstruction	1 (0.6)	3 (1.7)	0.626
Retention of urine	3 (1.9)	3 (1.7)	1.000
Cardiopulmonary	0 (0)	1 (0.6)	1.000
Re-operation	1 (0.6)	8 (4.5)	0.220

chemoradiotherapy. These all have proven to be risk factors for AL in rectal cancer patients[4-10].

Several studies[20-23] reported the effects of the use of rectal tubes on the occurrence of AL, but the results of a meta-analysis[4] indicated that there was no statistically significant association (OR = 0.48; 95%CI: 0.20-1.12, *P* = 0.09). Thus, it appears that postoperative AL cannot be reliably prevented by placement of a rectal tube only. Prophylactic ileostomy procedure has been widely adopted to prevent AL and its impact on decreasing this adverse outcome has been widely verified[11-14]. The rectal cancer surgery reported here tends to dissociate the rectum laparoscopically and extracts specimens *via* a small incision. In our study, we combined extracting the surgical specimen and performing the prophylactic ileostomy procedure at the outset. Therefore, the small incision was not employed. In theory, this approach embodies the benefits of minimally invasive surgery.

In our study, the primary results indicated that patients in the experimental group had a decreased incidence of wound infections (0% *vs* 4.0%, *P* = 0.016), and less pain during the first two postoperative days (2.6 ± 0.8 *vs* 3.1 ± 1.1, *P* < 0.001 on day 1; 1.6 ± 0.6 *vs* 1.8 ± 0.7, *P* = 0.012 on day 2) compared with the control group. In addition, compared with the patients in the control group, patients in the experimental group had shorter operative time (161.3 ± 21.5 min *vs* 168.8 ± 20.5 min, *P* = 0.001) and less estimated blood loss (77.4 ± 30.7 mL *vs* 85.9 ± 35.5 mL, *P* = 0.020). Although the stoma had larger average diameter in the experimental group than in the control group (4.7 ± 0.5 cm *vs* 4.0 ± 0.6 cm, *P* < 0.001), stoma-related complications (including infections, retractions, bleeding, and edema) were not increased. Four cases in the experimental group suffered from parastomal hernias, but they recuperated after the procedure to close the ileostomy. No patients suffered from positive distal margin complications or CRM in either group. Thus, the results of the present study support the safety and efficacy of this procedure.

In this study, we would like to emphasize that a single-use incision protector should be used to protect incision from pollution or cancer cell implantation when taking out the surgical specimen *via* the incision, and the ileostomy incision should not be too small so that the specimen is squeezed. It is also crucial to stress that, when suturing the skin and intestinal wall, the stoma should be constructed to avoid retraction (Figure 1A). In addition, the descending colon should be dissected and fully freed up to ensure enough length is available for re-anastomosis at the margin of the proximal resection.

Although the use of the ileostomy site as the specimen extraction site has already been described in patients with inflammatory bowel disease[24,25], the use in rectal cancer has not been reported. This was a retrospective study, in which all the laparoscopic procedures were performed by different surgeons independently, based on their own considerations, preferences and clinical judgement. Therefore, bias may exist. However, we have included a large sample and a long follow-up time. In addition, the two groups were well balanced in clinical characteristics and pathological findings which may have influenced the results. We hope that further randomized prospective controlled trials will be conducted to confirm our results in the near future.

In conclusion, surgical specimen extraction *via* a prophylactic ileostomy procedure represents a secure and feasible approach to laparoscopic rectal cancer surgery, and embodies the principle of minimally invasive surgery.

ARTICLE HIGHLIGHTS

Research background

A vertical or horizontal incision in the lower abdomen about 5 cm long was

utilized to extract the specimen, and then a circular incision about 4 cm in diameter was made in the right lower quadrant to complete the ileostomy for rectal cancer patients who accept prophylactic ileostomy. With ongoing developments in minimally invasive surgery, we tried an innovative method that involved surgical specimen extraction through a prophylactic ileostomy procedure so as to avoid making a vertical or horizontal incision in the lower abdomen. This procedure has not been reported in rectal cancer patients.

Research motivation

The purpose of this study was to compare and analyze the short and long-outcomes of surgical specimen extraction *via* a prophylactic ileostomy procedure *vs* a small lower abdominal incision procedure. The significance of this study is to inaugurate a more minimally invasive method for rectal cancer patients who accept prophylactic ileostomy.

Research objectives

The study aimed to evaluate the safety and feasibility of surgical specimen extraction *via* a prophylactic ileostomy procedure in patients with rectal cancer.

Research methods

Rectal cancer patients who accepted laparoscopic anterior resection and prophylactic ileostomy were systemically reviewed from June 2010 to October 2016 in our institution. Clinical characteristics, operative outcomes, pathological outcomes, postoperative complications, and follow-up information were collected and analyzed using SPSS version 21.0.

Research results

The results showed that mean operative time was significantly shorter in the experimental group compared to the control group ($P = 0.001$). Mean estimated blood loss was significantly less in the experimental group ($P = 0.020$). The pain reported by patients in the experimental group was significantly less than that of the controls during the first two days after surgery ($P < 0.001$ and $P = 0.012$, respectively). Postoperative complications did not increase. The estimated 5-year disease-free survival and overall survival rates were similar between the two groups ($P = 0.671$ and $P = 0.645$, respectively).

Research conclusions

Surgical specimen extraction *via* a prophylactic ileostomy procedure represents a secure and feasible approach to laparoscopic rectal cancer surgery, and embodies the principle of minimally invasive surgery.

Research perspectives

In this study, we would like to emphasize that a single-use incision protector should be used to protect incision from pollution or cancer cell implantation when taking out the surgical specimen *via* the incision, and the ileostomy incision should not be too small so that the specimen is squeezed. This was a retrospective study, and bias may exist. We hope that further randomized prospective controlled trials will be conducted to confirm our results in the near future.

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ORIGINAL ARTICLE

Prospective Study

Characterization of biofilms in biliary stents and potential factors involved in occlusion

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Author contributions: Vaishnavi C conceived the original idea and prepared the study design, provided administrative support, supervised the project and collected the data and finalized the manuscript; Kochhar R provided patients' samples and their clinical details; Samanta J analyzed the data statistically with the help of the department of Biostatistics and wrote the draft manuscript; Kochhar R critically revised the draft manuscript; all authors have directly contributed to the study, reviewed and approved the final manuscript for submission.

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Clinical trial registration statement: We have registered our clinical trial with the Indian Council of Medical Research, New Delhi, India, and gave them annual reports of the progress of the project on study of organisms responsible for biofilm formation in biliary stents and their molecular characterization.

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Abstract

AIM

To quantify the components in biofilms and analyze the predisposing factors involved in occlusion of biliary stents.

METHODS

In a prospective study conducted from April 2011 to March 2014 at a tertiary care hospital, all consecutive patients who required endoscopic biliary stent exchange/removal were included. Etiology of the biliary disease was diagnosed by imaging, cytology and on follow-up. Clinical details of patients with biliary stent retrieval were noted. All extracted stents were collected in sterile containers and immediately

processed for quantification of biofilm proteins and polysaccharides. Molecular identification of commonly known and unknown bacteria was performed by polymerase chain reaction and density gradient gel electrophoresis methods.

RESULTS

Eighty one patients (41 males) with age range of 20-86 years were studied. The underlying causes for stent insertion were bile duct stones (n = 46; 56.8%) benign stricture (n = 29; 35.8%) and malignancy (n = 6; 7.4%) with cholangitis in 50 (61.7%) patients. The retrieved stent sizes were 7 Fr (n = 62; 76.5%) and 10 Fr (n = 19; 23.5%) with 65 days median insertion duration. Polybacterial consortia were detected in 90.1% of the stents. The most common bacteria identified by polymerase chain reaction alone and/or sequencing were *Pseudomonas* (n = 38), *Citrobacter* (n = 23), *Klebsiella* (n = 22), *Staphylococcus* (n = 20), *Serratia* (n = 16), *Escherichia coli* (n = 14), *Streptococcus* (n = 13), *Enterococcus* (n = 13), *Aeromonas* (n = 12), *Proteus* (n = 10) and *Enterobacter* (n = 9). Protein concentration according to gender (0.547 ± 0.242 mg/mL *vs* 0.458 ± 0.259 mg/mL; P = 0.115) as well as age > 60 years and < 60 years (0.468 ± 0.295 mg/mL *vs* 0.386 ± 0.238 mg/mL; P = 0.205) was non-significant. However, polysaccharide concentration was significant both according to gender (0.052 ± 0.021 mg/mL *vs* 0.049 ± 0.016 mg/mL; P < 0.0001) and age (0.051 ± 0.026 mg/mL *vs* 0.038 ± 0.016 mg/mL; P < 0.011). Protein concentration in the biofilm was significantly higher (0.555 ± 0.225 mg/mL *vs* 0.419 ± 0.276 mg/mL; P = 0.018) in patients with cholangitis, lower (0.356 ± 0.252 mg/mL *vs* 0.541 ± 0.238 mg/mL; P = 0.005) in the 10 Fr group than the 7 Fr group, and significantly higher (0.609 ± 0.240 mg/mL *vs* 0.476 ± 0.251 mg/mL; P = 0.060) in stents of ⩾ 6 mo of indwelling time. However presence/absence of cholangitis, size of stent, indication of stent insertion and indwelling time did not affect the quantity of polysaccharide concentration.

CONCLUSION

Plastic stents retrieved from patients with biliary tract disease showed polymicrobial organisms with higher protein content among patients with cholangitis and those with smaller diameter stents. Longer indwelling duration had more biofilm formation.

Key words: Biofilm constituents; Polybacterial profile; Predisposing factors; Underlying causes; Biliary stents

Core tip: This prospective study evaluated the components in biofilms of retrieved biliary stents and analyzed predisposing factors involved in the process. A majority of stents showed growth of polymicrobial consortia. Polymerase chain reaction and sequencing helped to detect several microorganisms in most of the stents. Presence of cholangitis, smaller diameter of stents and longer indwelling time of stents were associated with higher chance of biofilm formation. To prevent stent occlusion, longer diameter stents with an indwelling time of 3 to 6 mo should be used.

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INTRODUCTION

Biliary strictures are responsible for severe complications which can be serious or life threatening to the patients[1]. Transpapillary endoscopic stent placement helps in the relief of obstructed biliary system by a non-surgical approach in patients with benign or malignant biliary disease[2,3]. The natural microbial barrier posed by the sphincter of Oddi is breached when a stent is placed across it and creates a low resistance pathway for colonization by the intestinal microbes[4]. Plastic biliary stents often get occluded by biofilms formed due to adhering microorganisms embedded in an exopolysaccharide (EPS) matrix[5]. A biofilm is defined as a collection of microbial communities enclosed by a matrix of EPS, separated by a network of open water channels and attached to man-made or natural surfaces. Bacterial biofilms are formed when unicellular bacteria come together to form a community that is attached to a solid surface and get encased in an exopolymeric substance largely comprising of proteins and different extracellular polymers[6]. The proposed mechanism of biofilm formation initiates with the process of priming of the stent surface with various proteins followed by microbial adherence and subsequently formation of an EPS matrix to embed the microbial colonies and other "foreign bodies" to give rise to the final mature biofilm[2,3]. Biofilms formed inside biliary stents consist of a mixed spectrum of bacterial communities[2]. Most of these bacteria, generally coming from the enterocolon, are uncultivable by standard culture methods.

Clinical stent occlusion leads to jaundice and bacterial cholangitis with polymicrobial infections in up to 90% of patients[7,8]. Improper use of antimicrobial agents against these microbes leads to antimicrobial resistance and consequently to ineffective treatment of stent-associated cholangitis[9]. Moreover occluded stents need repeat procedures and subsequently lead to increased medical costs as well as poor quality of life. Microorganisms isolated from blocked biliary stents include both aerobic and anaerobic species apart from fungi[2] and reveal their intestinal origin[10-12]. The material properties of the biofilm are heavily dependent on the composition of the EPS which consists of

proteins, polysaccharides, nucleic acids and lipids in varying proportions depending on the milieu in which the biofilm grows[13]. Materials derived from bacteria and the host form a conditioning film which lays the foundation for the biofilm development and initiates the process of bacteria-driven sludge formation[3].

In spite of multiple studies on isolation of various organisms in the formation of biofilms, factors involved in the formation of these biofilms are not well studied. Proper characterization of biofilm formation in plastic stents is yet to be adequately explained before steps for its prevention can be made successful. In this study we elucidated (1) the various bacteria in biofilm formation in biliary plastic stents by molecular identification inclusive of polymerase chain reaction (PCR) and sequencing; (2) principal constituents of biofilms *viz.* proteins and polysaccharides; and (3) the possible predisposing factors in relation to biofilm formation in the stents.

MATERIALS AND METHODS

Study population

This was a prospective study conducted over a three year period at a tertiary care hospital in Northern India (Postgraduate Institute of Medical Education and Research, Chandigarh, India) from April 2011 to March 2014. During this period, all consecutive patients who required an elective or emergency biliary stent exchange/removal were included in the study. The study was reviewed and approved by the Institutional Ethics Committee which operates according to the Declaration of Helsinki. Written informed consent was taken from all the patients prior to study enrollment. Clinical details of each patient were noted with reference to age, sex, etiology, presence of cholangitis and duration for which the stents had been *in situ*. Cholangitis was diagnosed as per the Tokyo guidelines[14]. Etiology of the biliary disease was diagnosed by imaging, cytology and on follow-up. All the stents had been placed endoscopically earlier in our institution.

Interventional procedure

Stent exchange or removal was carried out by first confirming the position of the stent under fluoroscopy. Thereafter, the stents were retrieved endoscopically after grasping with sterile foreign body forceps or a snare and withdrawal of the instrument wholly. The retrieved stents were immediately transferred to a sterile container and transported to the Microbiology Division of the department for processing in order to provide good pre-analytic conditions.

Molecular identification of the bacterial species occluding the biliary stents

For molecular identification of bacterial species, the central part of the biliary stents were cut and divided horizontally under sterile conditions. The encrusted sludge within the stent was then cultured aerobically in Brain Heart Infusion broth. For identification of anaerobic bacteria, the crust from stents were cultured in Brucella broth under anaerobic conditions. The microbial DNA was extracted from the culture growth by phenol-chloroform method. Briefly 1.5 mL media containing the growth was centrifuged at 12000 g for 10 mins. The supernatant was discarded and the pellet obtained was resuspended in Tris-EDTA (TE). Sodium dodecyl sulfate (0.5%) and proteinase K (200 μg/mL) was added and incubated for 30 min. Next 100 μL sodium chloride was added followed by an equal amount of chloroform-iso-amyl alcohol. The solution was mixed thoroughly and centrifuged. The supernatant was transferred to fresh tube and equal amount of phenol: chloroform: iso-amyl alcohol was added. After mixing again the solution was centrifuged and the supernatant obtained was transferred to another tube to which 0.6 volume of iso-propanol was added. Centrifugation was repeated and the pellet was washed with 70% ethanol. The pellet obtained was air dried, dissolved in TE and run in 0.8% agarose gel for checking for DNA.

Identification of commonly known bacteria involved in biofilm formation: PCR was standardized using the universal 16S rRNA gene specific primers for determining the DNA sequence for commonly known bacteria such as *Pseudomonas, Escherichia coli, Citrobacter, Streptococcus, Aeromonas, Enterococcus, Staphylococcus, Proteus, Bacillus, Klebsiella, Enterobacter, Serratia, Vibrio, Yersinia, Bacteroides* and *Clostridium* using standard strains obtained from Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India, as positive controls. Following standardization, PCR was done for identification of the above known bacteria that could be responsible for biofilm formation in the stents. The primers used for determination of bacteria are given in Table 1. The amplicons were visualized on 1.5% agarose gels stained with ethidium bromide and compared to a database of known sequences.

Identification of unknown bacteria involved in biofilm formation: Molecular identification of unknown bacteria involved in biofilm formation was done using the Density Gradient Gel Electrophoresis (DGGE). The DNA isolated from the biofilms were used for creating multiple copies of the 16S rRNA genes of similar but not identical bacteria for identifying unknown bacteria. The variable regions V3 to V5 were amplified using the following universal primers: 341-F (5′-CCT ACG GGA GGC AGC AG-3′) with a 40 bp GC sequence clamped to its 5′ end (5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3′) and 907-R (5′-CCG TCA ATT CMT TTG AGT TT-3′). This set of primers was designed to be specific for most bacteria[15]. The reaction mixture (50 μL) contained 50 ng

Table 1 Primer sequences used for polymerase chain reaction

Sr. No	Organism	Primer sequences
1	*Pseudomonas*	F: 5'-GACGGGTGAGTAATGCCTA- 3' R: 5'-CACTGGTGTTCCTTCCTATA -3'
2	*Staphylococcus*	F: 5'- AAC TCT GTT ATT AGG GAA GAA CA- 3' R: 5'- CCA CCT TCC TCC GGT TTG TCA CC-3'
3	*E. coli*	F: 5'-GAAGCTTGCTTCTTTGCT- 3' R: 5'-GAGCCCGGGGATTTCACAT- 3'
4	*Enterococcus*	F: 5'- GTTTATGCCGCATGGCATAAGAG -3' R: 5'-CCGTCAGGGGACGTTCAG -3'
5	*Citrobacter*	F: 5'-TCAGATTTGAACGCTGGCGGCA -3' R: 5'-CGTATTACCGCGGCTGCTGCCAC -3'
6	*Proteus*	F: 5'-AGA GTT TGA TCC TGG CTC AG-3' R: 5'-AAG GAG GTG ATC CAG CC-3'
7	*Klebsiella*	F: 5'-AGA GTT TGA TCC TGG CTC AG-3' R: 5'-AAG GAG GTG ATC CAG CC-3'
8	*Clostridium*	F: 5'-TGG CTC AGA TTG AAC GCT GGC GGC -3' R: 5'-TAC CTT GTT ACG ACT TCA CCA CA-3'
9	*Bacillus*	F:5'- AGA GTT TGA TCC TGG CTC AG -3' R: 5'- AAG GAG GTG ATC CAG CCG CA-3'
10	*Vibrio*	F: 5'-AGA GTT TGA TCA TGG CTC AG -3' R:5'-GAA ATT CTA CCC CCC TCT ACA G-3'
11	*Aeromonas*	F: 5'-GCT GGT CTG AGA GGA TGA TC-3' R: 5'-CTT TAC GCC CAG TAA TTC CG-3'
12	*Bacteroides*	F: 5'- ATT CTA GAG TTT GAT CAT GGC TCA-3' R: 5'-ATG GTA CCG TGT GAC GGG CGG TGT GTA-3'
13	*Enterobacter*	F: 5'-AGTTTGATCCTGGCTCAG -3' R: 5'-TAC CTT GTT ACG ACT TCG TCC CA-3'
14	*Streptococcus*	F: 5'-TAA CCA GAA AGG GAC GGC TA-3' R: 5'-CAC TCT CCG CTT CTG CAC TC-3'
15	*Serratia*	F : 5'-GCGGTTTGTTAAGTCAGATG -3' R: 5'-CGAATTAAACCACATGCTCC-3'
16	*Yersinia*	F : 5'-AAT ACC GCA TAA CGT CTT CG-3' R: 5'-CTT CTT CTG CGA GTA ACG TC-3'

microbial DNA, 200 μmol/L of each deoxynucleoside triphosphate, 0.5 pmol/L of each of the primers, 2.5 mmol/L $MgCl_2$, 3 mg/mL BSA and 3 U DNA Taq polymerase. The touchdown PCR was performed in eppendorf thermocycler using a program described by Sánchez *et al*[16]. Following an initial denaturation at 95 ℃ for 3 min, a touchdown program began with 15 cycles consisting of one minute denaturation at 95 ℃, one minute annealing beginning at 65 ℃ and ending at 50 ℃ (decreasing 1 ℃ per cycle), and a one minute extension at 72 ℃. A final extension of 5 min at 72 ℃ was done. PCR products were quantified on 1.5% (w/v) agarose gel. The desired PCR product was 594 bp (including the GC clamp).

The sample was loaded in the DGGE gel solution consisting of 6% (w/v) acrylamide/bisacrylamide (37.5:1) in 0.5 × TAE buffer containing 40% to 60% of the denaturant. The gels were prepared, loaded and run according to the instructions of the manufacturers of DGGE system (D-Code, BioRad, United States) for analysis of PCR products.

For sequencing, the selected DGGE bands were excised from the gels using sterile scalpel and placed in a sterile eppendorf containing 20 μL of sterile water. The amplified PCR products were sequenced commercially (Chromous Biotech, Bengaluru, India) using bands which were different from commonly known bands. Data obtained after sequencing in fasta format were compared with the National Center of Biotechnology Information (NCBI) GenBank data base using standard nucleotide blast search tools (BLAST N 2.2.29+).

Quantification of major molecules in the biofilms

The major molecules like proteins and carbohydrates which act as a molecular glue for biofilm formation were measured as follows.

Protein estimation: Protein estimation in the biofilm mass was done by modified Lowry's method as described by Raunkjær *et al*[17]. Briefly, one centimeter of the stent was put into a 1.5 mL centrifuge tube and 500 μL sodium hydroxide (0.5 mol/L) was added to it. The tube with the stent was heated at 80 ℃ for 30 min in a water bath. Centrifugation at 4238 *g* at 4 ℃ for 15 min was done and supernatant was transferred to another micro-centrifuge tube. 50 μL supernatant was put into a test tube and 1 mL reagent comprising of $CuSO_4 \cdot 5H_2O$ and sodium tartrate was added. It was incubated for 5 min at room temperature and absorbance was read at 620 nm using a colorimeter (Electronics India). Bovine serum albumin served as the standard for the assay.

Table 2 Organisms identified by polymerase chain reaction and sequencing (accession Nos. KP198519-43; KP205043-80; KP2)

Sr. No.	Organisms identified by touchdown PCR and sequencing	Organisms identified by PCR alone
BF 1	*Stenotrophomonas maltophilia*	*Klebsiella*
BF 2	*Pseudomonas stutzeri*	*Pseudomonas, Proteus, Aeromonas*
BF 3	*Bacillus tequilensis*	*Staphylococcus, Bacillus*
BF 4	Uncultured bacterium clone DolRC 17069	*Streptococcus, Aeromonas, Serratia*
BF 5	*Bacillus cereus*	*Staphylococcus, Bacillus*
BF 6	*Micrococcus yunnanensis*	*Streptococcus, Proteus, Serratia*
BF 7	*Staphylococcus epidermidis*	*Staphylococcus, Bacillus*
BF 8	*Citrobacter* sp.	*Citrobacter, Escherichia coli*
BF 9	*Stenotrophomonas maltophilia*	*Proteus*
BF 10	*Anaerosalibacter* sp.	*Pseudomonas, Citrobacter*
BF 11	*Enterobacteriales bacterium*	*Staphylococcus, Streptococcus, Klebsiella*
BF 12	Uncultured bacterium clone PS B346	*Staphylococcus, Aeromonas*
BF 13	*Stenotrophomonas maltophilia*	– –
BF 14	*Stenotrophomonas maltophilia*	*Streptococcus*
BF 15	*Stenotrophomonas maltophilia*	*Serratia*
BF 16	Uncultured organism clone ELU0026	*Citrobacter*
BF 17	Uncultured organism clone ELU0020	– –
BF 18	*Stenotrophomonas maltophilia*	*Citrobacter, Streptococcus*
BF 19	Uncultured bacterium clone ELU0020	*Klebsiella, Aeromonas, Enterococcus*
BF 20	*Bacillus mojavensis*	*Pseudomonas, Bacillus, Enterobacter*
BF 21	*Paenibacillus* sp. A1006	*Proteus, Klebsiella, Serratia*
BF 22	*Bacillus cereus*	*Streptococcus, Serratia*
BF 23	*Stenotrophomonas maltophilia*	– –
BF 24	*Bacillus* sp.	*Proteus, Yersinia, Aeromonas*
BF 25	*Pseudomonas stutzeri*	*Pseudomonas, Escherichia coli*
BF 26	*Enterobacteriales bacterium*	*Pseudomonas, Proteus, Klebsiella*
BF 27	Uncultured bacterium clone DolRc DL35rect19C08	*Proteus, Klebsiella*
BF 28	*Enterococcus faecalis*	*Citrobacter, Enterococcus*
BF 29	*Micrococcus luteus*	– –
BF 30	*Staphylococcus epidermidis*	*Staphylococcus, Serratia*
BF 31	*Staphylococcus epidermidis*	*Citrobacter, Escherichia coli, Staphylococcus*
BF 32	*Enterococcus durans*	*Escherichia coli, Klebsiella, Enterococcus*
BF 33	*Enterococcus durans*	*Staphylococcus, Streptococcus, Serratia*
BF 34	*Stenotrophomonas maltophilia*	*Pseudomonas*
BF 35	Uncultured bacterium clone B64	*Citrobacter, Proteus, Klebsiella*
BF 36	*Pseudomonas otitidis*	*Pseudomonas, Aeromonas*
BF 37	*Enterobacteriales bacterium*	*Streptococcus, Klebsiella, Enterobacter*
BF 38	*Pseudomonas alcaligenes*	*Citrobacter, Escherichia coli, Yersinia*
BF 39	*Enterococcus faecalis*	*Citrobacter, Streptococcus, Klebsiella*
BF 40	*Enterococcus faecalis*	*Streptococcus, Klebsiella , Aeromonas*
BF 41	*Enterococcus* sp.	*Citrobacter, Enterobacter*
BF 42	*Bacillus subtilis*	*Pseudomonas, Streptococcus, Aeromonas*
BF 43	*Pseudomonas aeruginosa*	*Pseudomonas, Escherichia coli, Yersinia*
BF 44	Uncultured bacterium	*Pseudomonas, Citrobacter, Yersinia*
BF 45	Uncultured bacterium	*Pseudomonas, Citrobacter, Escherichia coli*
BF 46	*Pseudomonas* sp.	*Escherichia coli, Enterobacter*
BF47	*Bacillus cereus*	*Proteus, Klebsiella*
BF 48	*Stenotrophomonas maltophilia*	*Pseudomonas, Citrobacter, Streptococcus*
BF 49	*Stenotrophomonas maltophilia*	*Pseudomonas, Staphylococcus, Proteus*
BF 50	*Bacillus cereus*	*Pseudomonas , Staphylococcus*
BF 51	*Pseudomonas aeruginosa*	*Pseudomonas, Citrobacter, Escherichia coli*
BF 52	*Pseudoxanthomonas icgebensis*	*Citrobacter, Serratia*
BF 53	*Enterobacteriales bacterium*	*Klebsiella, Enterobacter, Vibrio*
BF 54	*Citrobacter freundii*	*Citrobacter, Staphylococcus*
BF 55	Uncultured bacterium clone PS	*Staphylococcus, Serratia*
BF 56	*Enterobacteriales bacterium*	*Pseudomonas, Citrobacter, Streptococcus*
BF 57	*Morganella morganii*	– –
BF 58	*Pseudomonas aeruginosa*	*Pseudomonas, Escherichia coli, Staphylococcus*
BF 59	*Pseudomonas* sp.	*Pseudomonas, Staphylococcus, Serratia*
BF 60	*Pseudomonas putida*	*Pseudomonas, Klebsiella , Aeromonas*
BF 61	*Morganella morganii*	– –
BF 62	*Enterobacteriales bacterium*	*Pseudomonas, Aeromonas*
BF 63	*Enterococcus faecalis*	*Enterococcus*
BF 64	*Pseudomonas* sp.	*Pseudomonas, Staphylococcus, Serratia*
BF 65	*Bacillus* sp.	*Citrobacter, Enterobacter*
BF 66	Uncultured organism clone	– –
BF 67	*Pseudomonas sp.*	*Pseudomonas*

BF 68	*Bacillus licheniformis*	*Pseudomonas, Enterobacter*
BF 69	*Enterococcus* sp.	*Klebsiella , Serratia*
BF 70	*Enterococcus faecalis*	*Citrobacter, Enterobacter, Serratia*
BF 71	*Pseudomonas stutzeri*	*Pseudomonas, Staphylococcus, Aeromonas*
BF 72	*Escherichia coli*	*Escherichia coli*
BF 73	*Klebsiella* sp.	*Pseudomonas, Klebsiella*
BF 74	Uncultured *Klebsiella* sp.	*Escherichia coli*
BF 75	*Enterobacteriales bacterium*	*Escherichia coli, Enterobacter, Yersinia*
BF 76	*Stenotrophomonas maltophilia*	– –
BF 77	*Enterobacteriales bacterium*	*Klebsiella*
BF 78	*Citrobacter* sp. enrichment clone	*Citrobacter*
BF 79	*Serratia marcescens*	*Staphylococcus, Serratia*
BF 80	*Klebsiella* sp. BAB-3527	*Klebsiella, Aeromonas*
BF 81	*Exiguobacterium aurantiacum*	*Klebsiella*

PCR: Polymerase chain reaction.

Polysaccharide estimation: Polysaccharide estimation in the biofilm mass was done using the anthrone method as described by Ahimou *et al*[13]. Briefly, one centimeter of biliary stent was put into 1.5 mL centrifuge tube and 500 μL sodium hydroxide (1 N) was added to it and heated at 80 ℃ for 30 min in a water-bath. Centrifugation was done at 4238 *g* and 500 μL supernatant was transferred to another micro-centrifuge to which 500 μL distilled water and 4 mL of 0.2% anthrone reagent in concentrated sulfuric acid was added and mixed well. It was incubated for 10 min in boiling water-bath and allowed to cool at room temperature. Glucose (1 mg/10 mL) was used as a standard and absorbance was read at 620 nm using a colorimeter (Electronics India).

Statistical analysis

Statistical analysis for this study was performed using SPSS version 20.0 (IBM Corp., United States). The distribution of quantitative and qualitative data was presented as median (range) or absolute and relative frequencies. χ^2 test and Fisher's exact test were used to investigate the relationship between each parameter. Significance was defined as a P value < 0.05.

RESULTS

Patient and stent characteristics

A total of 81 patients (41 males) with age-range of 20-86 years were included in the study. The underlying causes for stent insertion were bile duct stones (n = 46, 56.8%) benign stricture (n = 29, 35.8%), and malignant stricture (n = 6, 7.4%). All the stents were double pig-tailed and made of polyethylene (Wilson-Cook Medical, Ireland) and had been placed endoscopically at our Institute. The diameter of the stents retrieved was 7Fr (n = 62, 76.5%) or 10 Fr (n = 19, 23.5%). The median duration of stent insertion was 65 days (range 5-1095 d). Cholangitis was present in 50 (61.7%) patients, at the time of stent insertion.

Constituents of biofilms

Microbiological analysis: Of the 81 stents retrieved, organisms were detected in 73 by PCR alone, whereas all 81 stents had organisms detected by touchdown PCR and sequencing which included uncultured bacteria in 12 stents (Table 2). Polybacterial consortia were detected in majority of the stents (n = 73, 90.1%) whereas single species were found in the remaining 8 (9.9%) stents. The most common Gram-negative bacteria detected by both PCR alone and by sequencing were *Pseudomonas* (n = 38), *Citrobacter* (n = 23), *Klebsiella* (n = 22), *Serratia* (n = 16), *Escherichia coli* (n = 14), *Aeromonas* (n = 12), *Proteus* (n = 10), *Enterobacter* (n = 9). The most prevalent Gram-positive bacteria were *Staphylococcus* sp. (n = 20) *Streptococcus* (n = 13) and *Enterococcus* (n = 13). Figure 1A and B show amplification of 541 bp of *Pseudomonas* sp and 500 bp of *Citrobacter* sp. as representative bacteria detected by PCR. Table 3 depicts the number of biliary stents in which Gram negative and Gram positive bacteria were detected.

Quantification of protein and polysaccharide in the biofilms: Protein content in the biofilm formation ranged from 0 to 0.98 mg/mL with a mean of 0.50 ± 0.25 mg/mL (Figure 2) while the polysaccharide content ranged from 0.014 to 0.107 mg/mL with a mean of 0.051 ± 0.018 mg/mL (Figure 3).

Relationship of biofilm constituents with predisposing factors

The relation of biofilm constituents with various predisposing factors was analyzed and is summarized in Table 4.

Gender and age groups: Male gender had higher protein concentration (P = 0.115) and polysaccharide concentration (P < 0.0001) than female gender. Patients > 60 years of age had higher protein concentration (P = 0.205) and polysaccharide concentration (P < 0.011) than those < 60 years of age.

Cholangitis: The quantity of biofilm components was compared in the stents retrieved from patients with (n = 50) and without cholangitis (n = 31). Protein

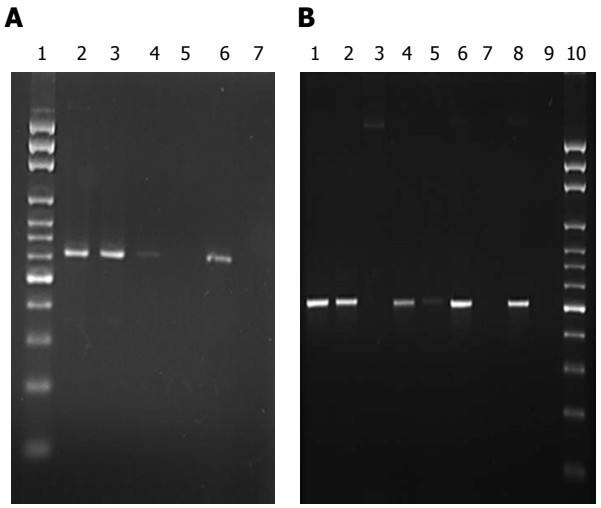

Figure 1 16S rRNA gene (541 bp) of *Pseudomonas* (A) and 16S rRNA gene (500 bp) of *Citrobacter* (B). A: Lane 1: DNA ladder 100 bp; Lanes 2-5: Samples; Lane 6: Positive control; Lane 7: Negative control. B: Lanes 1-7: Samples; Lane 8: Negative control; Lane 9: Positive control; Lane 10: DNA ladder 100 bp.

Table 3 Microbial species detected from the biliary stents (*n* = 81)

	Number of stents positive for the isolates
Gram-positive microorganism	
Bacillus sp.	11
Enterococcus sp.	9
Micrococcus sp.	2
Streptococcus sp.	11
Staphylococcus sp.	17
Gram-negative microorganism	
Citrobacter sp.	20
Escherichia coli	12
Enterobacter sp.	9
Klebsiella sp.	19
Morganella morganii	2
Proteus sp.	10
Pseudomonas sp.	27
Serratia sp.	15
Stenotrophomonas maltophila	11
Vibrio sp.	1
Yersinia sp.	5
Aeromonas sp.	12

concentration was found to be significantly higher (*P* = 0.018) in stents with cholangitis (0.555 mg/mL) as compared to those without cholangitis (0.419 mg/mL). Polysaccharide content was however not different in patients with or without cholangitis.

Indication of stent insertion: Biofilm constituents were also compared with etiology of biliary disease (benign stricture *vs* stone). However there was no statistical significance observed between CBD stone or benign stricture as regards to both protein and polysaccharide quantity.

Size of indwelling stents: Biofilm constituents were analyzed between two stent size groups of 7 Fr *vs* 10 Fr. Protein concentration in the 10 Fr group was significantly lower than in the 7 Fr group (0.356 ± 0.252 mg/mL *vs* 0.541 ± 0.238 mg/mL, *P* = 0.005). However there was no significant difference in the quantity of polysaccharide concentration (*P* = 0.674) in the stents of the two different sizes.

Duration of indwelling stents: When the stents with an indwelling time of ⩾ 3 mo were compared with those < 3 mo, it was found that there was no significant difference in the protein concentration (*P* = 0.472) or polysaccharide concentration (*P* = 0.385) between the two groups. When the stents with an indwelling time of ⩾ 6 mo were compared with those < 6 mo, it was found that protein concentration was significantly higher in the stents of ⩾ 6 mo of indwelling time (0.609 ± 0.240 mg/mL *vs* 0.476 ± 0.251 mg/mL, *P* = 0.060), but there was no difference in polysaccharide concentration (*P* = 0.560).

Number of microorganisms detected: When the number of microorganisms isolated *i.e.* single *vs* multiple by PCR alone was analyzed no significant difference was seen with respect to the protein (*P* = 0.996) and the polysaccharide parameters (*P* = 0.968).

GenBank submission

Most of the annotated DNA sequences obtained by sequencing from biofilms of each biliary stent have been deposited with the GenBank at NCBI, United States (Accession Nos. KP198519-43; KP205043-80; KP212173-77).

DISCUSSION

Biofilm formation is an important step in the occlusion of biliary stents and depends on a number of factors, inclusive of bacterial colonization[2,3]. Swidsinski *et al*[18] had demonstrated that neither the gall bladder wall nor the bile duct wall had any biofilm, denoting that opportunistic attachment of the microbes occurs later with subsequent biofilm formation on the biliary stents. In the natural setting, bacteria composed of a single species are seldom found in biofilms and most of the biofilms are multispecies consortia with a synergistic effect on the biofilm formation[19]. Aerobic *Enterococcus, E. coli* and *Klebsiella* as also anaerobic *Clostridia* are the most common microorganisms isolated from biliary sludge[2,20]. In our study, polybacterial consortia were seen in 90.1% of the biliary stents with most common microorganisms being *Pseudomonas, Citrobacter, Klebsiella,* and *Staphylococcus*. Similar frequency of polymicrobial consortia was found in patients with or without cholangitis. Schneider *et al*[20] also reported that occluded stents have higher proportion of *Staphylococcus* sp. as compared to the non-occluded ones. Lübbert *et al*[9] reported that enterococci plays a

Table 4 Various factors in relation to protein and polysaccharide concentrations

Parameters	Protein (mg/ml)	*P* value	Polysaccharide concentration (mg/mL)	*P* value
Gender				
Male (n = 41)	0.547 ± 0.242	0.115	0.052 ± 0.021	< 0.0001
Female (n = 40)	0.458 ± 0.259		0.049 ± 0.016	
Age				
Below 60 (n = 60)	0.386 ± 0.238	0.205	0.038 ± 0.016	0.011
Above 60 (n = 21)	0.468 ± 0.295		0.051 ± 0.026	
Etiology of stenting				
Cholangitis (n = 50)	0.555 ± 0.225	0.018	0.0512 ± 0.021	0.790
No cholangitis (n = 31)	0.419 ± 0.276		0.050 ± 0.014	
Indication of stent insertion				
CBD stone (n = 46)	0.518 ± 0.256	0.530	0.051 ± 0.022	0.785
Benign stricture (n = 29)	0.453 ± 0.256		0.050 ± 0.012	
Indwelling stent size				
7 Fr (n = 62)	0.541 ± 0.238	0.005	0.049 ± 0.015	0.674
10 Fr (n = 19)	0.356 ± 0.252		0.052 ± 0.020	
Duration of indwelling stents				
< 3 mo (n = 39)	0.481 ± 0.242	0.472	0.0489 ± 0.015	0.385
⩾ 3 mo (n = 42)	0.523 ± 0.264		0.0525 ± 0.022	
< 6 mo (n = 65)	0.476 ± 0.251	0.060	0.0501 ± 0.017	0.560
⩾ 6 mo (n = 16)	0.609 ± 0.240		0.0533 ± 0.026	
No of microorganisms detected monomicrobial (n = 13)	0.502 ± 0.263	0.996	0.051 ± 0.018	0.968
Polymicrobial (n = 68)	0.501 ± 0.050		0.049 ± 0.015	

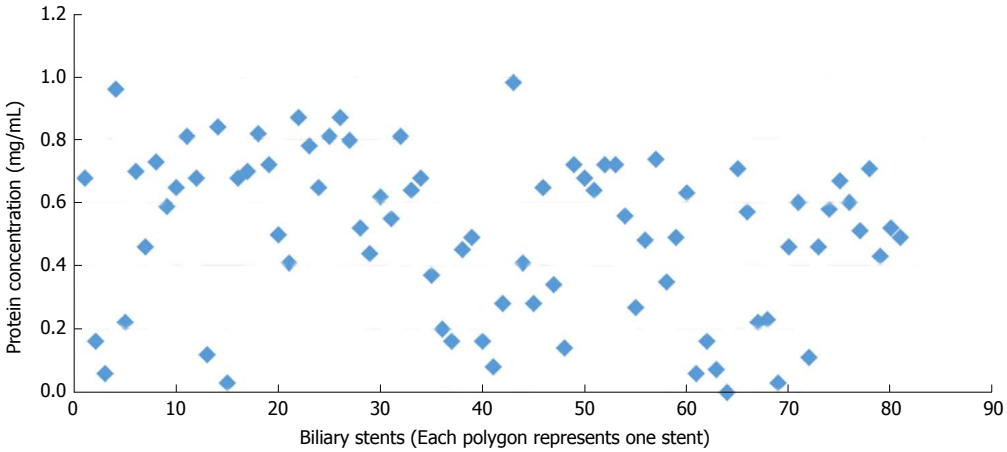

Figure 2 Protein concentration in biofilms of the biliary stents (n = 81).

significant part in the microbial colonization of biliary stents. In our study enterococci were found in 16% of the biliary stents. Though anaerobes are reported by some authors to have important role in the formation of biofilms[20], we found no anaerobe in the occluded biliary stents in the present study.

The proposed mechanism of biofilm formation is initiated with the process of priming of the stent surface with various proteins followed by microbial adherence and subsequent formation of an EPS matrix to embed the microbial colonies and other particles to give rise to the final mature biofilm. Yu *et al*[21] reported attachment of fibronectin to the inner surface of the stents within 24 h of exposure to bile. Another contributing factor is the bile immunoglobulin-bacteria complex which further promotes the binding of the bacteria to the inner surface of the stents[22]. Thus, the basic ingredients of a biofilm include the adherence proteins, the bacteria and the EPS. In patients with cholangitis, wherein these factors are expected to be high, there are higher chances of biofilm formation and stent occlusion. In the current study, the protein concentration of the biofilms was found to be significantly higher in stents placed in patients with cholangitis than those without cholangitis. Polysaccharide concentration was also higher among the cholangitis group, although it was not statistically significant. This highlights the phenomenon of higher propensity of stent occlusion due to biofilm formation in an infected biliary system as compared to the non-infected ones. The higher risk of stent occlusion in

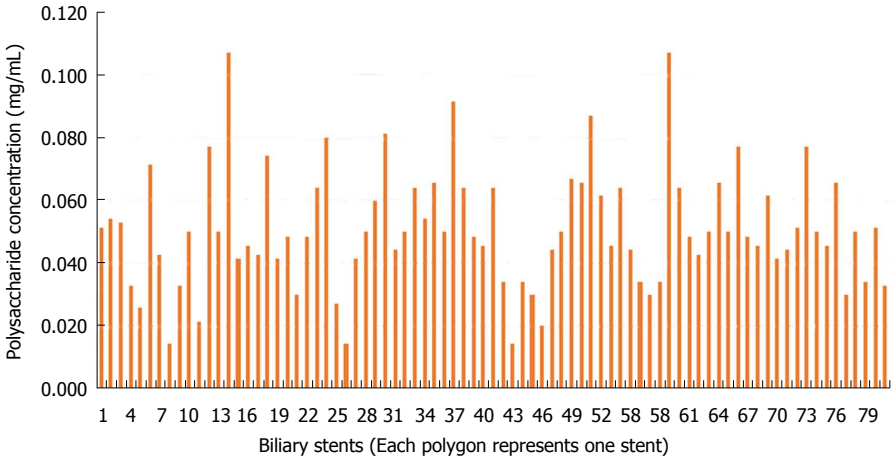

Figure 3 Polysaccharide concentration in biofilms of the biliary stents (*n* = 81).

cholangitis can also be explained by the increased bile viscosity of the infected bile, causing decrease in the bile flow velocity leading to bile stasis and increased spontaneous and bacteria-driven bile salt precipitation[2].

The diameter of the biliary stents has always been a key issue governing the dynamics of bile flow and stent occlusion. An increase in the inner stent diameter of 0.2 mm leads to a 300% increase in the bile flow[23]. The maximum diameter of plastic stent that can be placed endoscopically is 11.5 Fr[24]. This limitation of the maximum diameter of stents placed endoscopically is the reason why stents up to 10 Fr size are used. Smaller diameter stents have a higher tendency to get occluded due to biofilm formation. Larger diameter entails greater bile flow velocity and subsequently less predisposition to bile salt precipitation, protein accumulation and biofilm formation. Thus, large diameter stents have always fared better than smaller diameter ones in terms of durability[25] and one of the major advantages of metallic stents is in fact its large diameter[26]. In the current study, 10 Fr stents were found to have significantly lower protein concentration in their biofilm formation as compared to the 7 Fr ones. This highlights a probable lower propensity for protein deposition - one of the key events for initiation of biofilm formation due to high bile flow velocity in the 10 Fr groups.

The process of biofilm formation is a time-dependent one and risk of standard polyethylene stent occlusion increases progressively after 3 mo[2]. We did not find any difference in the protein and polysaccharide concentration in relation to stents removed < 3 mo and ⩾ 3 mo. However when the stents placed for < 6 mo were compared with those of ⩾ 6 mo, the protein concentration was found to be higher in stents kept for ⩾ 6 mo. The nature of protein (human/bacterial origin, immunoglobulins, fibrinogen *etc*.) could also not be further analyzed as this was not the aim of the study. Quantitative assessment of the number of bacteria present in the stents could not be made as molecular identification was carried out after culture of bacteria from the stent segments in fluid culture media. The process of biofilm formation in general is very complex (obstruction of biliary stents is more complex and involves not only bacteria and their products but bilirubin complexes, cholesterol complexes and ingrowth of tissue). Schneider *et al*[20] in a multivariate analysis have shown that sludge formation had significant relationship with stent indwelling time.

Understanding pathophysiology of biofilm formation in plastic biliary stents is important in preventing their occlusion and complications thereof. From our data stents indwelling time of ⩽ 3 mo or > 3 mo did not correlate with biofilm formation. However stents placed for > 6 mo had higher biofilm formation. Hence stents should not be left indwelling beyond 6 mo. Also larger diameter stents (10 Fr) should be preferred. A number of other options have been studied to prevent biofilm formation. Several studies have shown the effects of antibiotic coatings on medical devices effective against biofilm formation; however, such data has not been as successfully replicated for biliary stents[27]. Recently some workers have found that biliary plastic stents coated with silver nano particles or ions have antibacterial activity against several organisms and extends the period of use of biliary stents[28,29].

Our study had a few limitations. Data on comorbidities were not available, so we could not study the predisposition, if any, of biofilm formation in patients with diabetes. We had only a few patients with malignancy who were excluded from analysis. A larger number of patients with malignant obstruction could have given us a comparison between benign and malignant etiology. Culture results of patients with cholangitis at the time of stent insertion were not available to correlate with organisms grown in the biofilms.

Ours is one of the first studies of its kind to measure the biofilm components, namely protein and

polysaccharide in biliary stents. Presence of cholangitis at the time of stent insertion and smaller diameter of stents were found to have higher protein concentration, whereas male gender and age above 60 years had higher polysaccharide concentration, predisposing to higher propensity of biofilm formation. Longer (⩾ 6 mo) indwelling time of stents was associated with higher biofilm formation and protein concentration elucidating the time-dependent process of biofilm formation. Our data suggest that plastic stents should be replaced between 3-6 mo.

ARTICLE HIGHLIGHTS

Research background

Since its introduction in 1979, biliary plastic stents have been a landmark achievement in the field of endoscopic retrograde cholangiopancreatography for the relief of obstructed biliary system by a non-surgical approach. The limiting factors for these plastic stents are their diameter and the tendency to get occluded. The maximum diameter of plastic stent that can be placed is 11.5 Fr requiring a duodenoscope accessory channel diameter of 4.2 mm. This limitation of the maximum diameter leads to the tendency for them to get occluded due to the formation of biofilm causing recurrent obstruction and need for repeat procedures subsequently leading to increased medical costs and poor quality of life. The cardinal step in the process of stent occlusion is bacterial colonization. Various studies including scanning electron microscopic observations have shown that the clogging material found in biliary stents consists of bacterial biofilm, biliary sludge and duodenal refluxate of dietary fibers. Biofilm is formed by microbes embedded in an exopolysaccharide matrix which also engulfs "foreign bodies" of various sizes. Its ultrastructure reveals voids and channels required for nutrient diffusion and molecular signaling.

Research motivation

Despite multiple studies elucidating the various organisms and the formation of biofilms, various factors involved in the formation of these biofilms are not well studied. The proposed mechanism of biofilm formation initiates with the process of priming of the stent surface with various proteins followed by microbial adherence and subsequently formation of an exopolysaccharide matrix to embed the microbial colonies and other "foreign bodies" to give rise to the final mature biofilm. However, proper characterization of biofilm formation in plastic stents has to be adequately elucidated before steps for its prevention can be made successful. Components of the biofilm such as protein and polysaccharides developing in biliary stents have never been quantified in previous studies.

Research objectives

The main objectives of this study were to elucidate the various bacteria implicated in biofilm formation in biliary plastic stents, to quantify the principal constituents (namely proteins and polysaccharide) of biofilm mass and the possible predisposing factors in relation to biofilm formation in the stents. This prospective study evaluated the extracellular polymeric substance such as protein and polysaccharide in the biofilms as well as microbes occluding the biliary stents in patients who had retrieval of biliary stents (7 Fr and 10 Fr) and analyzed predisposing factors involved in the process of occlusion of the stents. Our results showed that the presence of cholangitis at the time of stent insertion and smaller diameter of stents were found to have higher protein concentration, whereas male gender and age above 60 years had higher polysaccharide concentration, predisposing to higher propensity of biofilm formation. Longer (⩾ 6 mo) indwelling time of stents was associated with higher biofilm formation and protein concentration, elucidating the time-dependent process of biofilm formation. Our data suggest that plastic stents should be replaced between 3-6 mo. Further studies can be done to explore the origin of the bacteria grown in biofilms. Strategies to prevent biofilm formation can also be planned and investigated.

Research methods

This was a prospective study conducted at a tertiary care hospital in Northern India (Postgraduate Institute of Medical Education and Research, Chandigarh, India) from April 2011 to March 2014. All consecutive patients who required an elective or emergency biliary stent exchange/removal were enrolled and clinical details of each patient were noted. The stents were retrieved through video duodenoscope and transferred into sterile containers for processing. For molecular identification of bacterial species, the encrusted material enclosed within the stent was cultured aerobically and anaerobically and the microbial DNA was extracted. PCR was standardized using the universal 16S rRNA gene-specific primers for determining the DNA sequence for commonly known bacteria. Molecular identification of unknown bacteria involved in biofilm formation was done using the Density Gradient Gel Electrophoresis. The amplified PCR products were sequenced commercially using bands which were different from commonly known bands. Data obtained after sequencing were compared with the National Center of Biotechnology Information GenBank data base, using standard nucleotide blast search tools. The major molecules in the biofilms such as protein and polysaccharide were estimated in the biofilm mass by modified Lowry's method and anthrone method respectively. The outcome measures were quantification of biofilm protein, polysaccharides and the organisms and their relation with gender, age, etiology of biliary diseases, stent indwelling time, stent size and the presence of cholangitis. Statistical analysis for this study was performed using SPSS version 20.0 using χ^2 test and Fisher's exact test to investigate the relationship between various parameters.

Research results

Higher protein concentration in the biofilm was noted in patients with cholangitis as compared to those without cholangitis. Cholangitis and protein concentration increased the likelihood of biofilm formation in these patients explaining higher stent occlusion rates in infected bile. Male gender and age above 60 years had higher polysaccharide concentration, predisposing to higher propensity of biofilm formation. Smaller diameter stents depicted higher protein concentration predisposing to early biofilm formation thereby indicating the use of larger diameter stents. 10 Fr stents had lower concentration of protein deposition in the biofilm compared to 7 Fr stents and hence explains the longer patency rates. PCR and sequencing helped to detect several commonly known and unknown microorganisms in most of the stents. Time dependent process of biofilm formation was demonstrated by greater quantity of biofilm mass deposition on increasing length of stent indwelling time. Longer indwelling time of stents has a greater likelihood of accumulating higher biofilm formation and patients should be followed-up between 3-6 mo to avoid complications.

Research conclusions

Presence of cholangitis at the time of stent insertion and smaller diameter of stents were found to have higher protein concentration, whereas male gender and age above 60 years had higher polysaccharide concentration, predisposing to higher propensity of biofilm formation. Longer indwelling time of stents was associated with higher biofilm formation and protein concentration elucidating the time-dependent process of biofilm formation. Our study suggests that plastic stents should be replaced between 3-6 mo. Plastic stents retrieved from patients with biliary tract disease showed polymicrobial organisms with higher protein content among patients with cholangitis and those with smaller diameter stents. Longer indwelling duration had more biofilm formation. Presence of cholangitis at the time of stent insertion and smaller diameter of stents were found to have higher protein concentration, whereas male gender and age above 60 years had higher polysaccharide concentration, predisposing to higher tendency of biofilm formation. Longer indwelling stent duration was associated with higher biofilm formation and protein concentration, revealing the time-dependent progression of biofilm formation. Longer indwelling time of stents, smaller diameter stents, male gender and age above 60 years are associated with more biofilm formation. Data on comorbidities such as diabetes in patients should be checked for predisposition, if any, of biofilm formation. Culture results of patients with cholangitis at the time of stent insertion will help to correlate with organisms grown in the biofilms. Protein concentration in the biofilm was significantly higher in patients with cholangitis, lower in the 10 Fr group than the 7 Fr group, and significantly higher in stents of ⩾ 6 mo of indwelling time. Polysaccharide concentration in biofilms of stents of male gender as well as

in patients with age > 60 years was significant. The most common bacteria identified by PCR alone and/or sequencing were *Pseudomonas* (*n* = 38), *Citrobacter* (*n* = 23), *Klebsiella* (*n* = 22), *Staphylococcus* (*n* = 20), *Serratia* (*n* = 16), *Escherichia coli* (*n* = 14), *Streptococcus* (*n* = 13), *Enterococcus* (*n* = 13), *Aeromonas* (*n* = 12), *Proteus* (*n* = 10) and *Enterobacter* (*n* = 9). Longer indwelling time of stents and smaller diameter stents are associated with more biofilm formation. Larger diameter (10 Fr) stents should be preferred for the relief of obstructed biliary system by a non-surgical approach in patients with benign or malignant biliary disease. Stents should not be kept *in-situ* for more than 3-6 mo.

Research perspectives

Our study suggests that 10 Fr stents should be preferred over the 7 Fr stents and stents should be replaced between 3-6 mo. Attempts to prevent biofilm formation should be investigated. Ultrastructural characterization of biofilms in occluded stents should also be done.

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SYSTEMATIC REVIEWS

Systematic review of colorectal cancer screening guidelines for average-risk adults: Summarizing the current global recommendations

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Abstract

AIM

To summarize and compare worldwide colorectal cancer (CRC) screening recommendations in order to identify similarities and disparities.

METHODS

A systematic literature search was performed using MEDLINE, EMBASE, Scopus, CENTRAL and ISI Web of knowledge identifying all average-risk CRC screening guideline publications within the last ten years and/or position statements published in the last 2 years. In addition, a hand-search of the webpages of National Gastroenterology Society websites, the National Guideline Clearinghouse, the BMJ Clinical Evidence website,

Google and Google Scholar was performed.

RESULTS

Fifteen guidelines were identified. Six guidelines were published in North America, four in Europe, four in Asia and one from the World Gastroenterology Organization. The majority of guidelines recommend screening average-risk individuals between ages 50 and 75 using colonoscopy (every 10 years), or flexible sigmoidoscopy (FS, every 5 years) or fecal occult blood test (FOBT, mainly the Fecal Immunochemical Test, annually or biennially). Disparities throughout the different guidelines are found relating to the use of colonoscopy, rank order between test, screening intervals and optimal age ranges for screening.

CONCLUSION

Average risk individuals between 50 and 75 years should undergo CRC screening. Recommendations for optimal surveillance intervals, preferred tests/test cascade as well as the optimal timing when to start and stop screening differ regionally and should be considered for clinical decision making. Furthermore, local resource availability and patient preferences are important to increase CRC screening uptake, as any screening is better than none.

Key words: Guidelines; Systematic review; Fecal occult blood test; Fecal immunochemical test; Colonoscopy; Colorectal cancer; Screening; Flexible sigmoidoscopy

Core tip: To our knowledge, this is the first systematic review comparing global colorectal cancer (CRC) screening guidelines for average risk individuals, aiming to highlight similarities and discuss areas of controversy. It is well established that screening reduces CRC incidence and mortality, however there are regional differences when it comes to implementing such screening. Moreover, several guidelines have been published or updated recently. Our review showed that average-risk individuals should undergo CRC screening from age 50 to 75, using guaiac-based fecal occult blood test, fecal immunochemical test, flexible sigmoidoscopy or colonoscopy.

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INTRODUCTION

According to the World Health Organization, colorectal cancer (CRC) is the third most common cancer diagnosed among men, and the second most common in women[1]. CRC screening of average-risk individuals decreases CRC incidence and mortality. Available CRC screening modalities include fecal occult blood testing (FOBT) that can either be guaiac-based (gFOBT) or immunochemical (FIT). Research including randomized controlled trials has shown that annual FOBT reduces CRC mortality by approximately 30%[2-8], whilst both annual and biennial FOBT screenings reduce CRC incidence[9]. However, those reductions can be obtained only if a positive FOBT is followed by more invasive investigations such as colonoscopy. Flexible sigmoidoscopy (FS) has shown to decrease CRC incidence by 30% and CRC-related mortality by 50%[10]. Colonoscopy is often referred to as the CRC screening gold standard because it allows an examination of the complete colon and it can remove pre-cancerous polyps immediately. However, whilst randomized controlled trials (RCTs) demonstrated that FS screening reduces CRC incidence and mortality[10,11], similar high-quality evidence is lacking for screening colonoscopy. Other potential screening methods include double contrast barium enema (DCBE), CT colonography, video capsule colonoscopy and stool DNA (sDNA) testing. However, their exact respective roles in CRC screening remain even less well recognized. Several guidelines on CRC screening have recently been updated[12-15]. This systematic review provides an overview over the current guidelines and discusses areas of uncertainty and controversy amongst them.

MATERIALS AND METHODS

Search strategy

Computerized medical literature searches were initiated from January 2007 to September 2017 using MEDLINE, EMBASE, Scopus, CENTRAL and ISI Web of knowledge. The selection of articles utilized a combination of MeSH headings and controlled vocabulary adapted to each databases related to (1) colorectal cancer; and (2) guideline (or recommendations, or position statement or consensus). Recursive searches and cross-referencing were also carried out using a "similar articles" function; hand searches of articles were identified after an initial search. We included all fully published adult human studies in English. In addition, we performed a hand-search of the webpages of National Gastroenterology Society websites (the complete list of screened societies is available in Appendix 1), the National Guideline Clearinghouse, the BMJ Clinical Evidence website, and Google and Google Scholar to identify relevant publications. Two authors independently performed searches, with a third available to resolve disagreements in citation selection.

Trial selection and study population

Selection criteria included guidelines, consensus recommendations or position statements that include specific recommendations for CRC screening in average-risk (asymptomatic, with no personal nor

family history) individuals. Exclusion criteria were: Guidelines (or consensus) publications older than ten years, position statements older than 2 years, articles reporting only on national colorectal screening programs [*i.e.*, Association of Coloproctology of Great Britain and Ireland (ACPGBI)] without issues actual guideline or consensus recommendations, and articles that were only reviewing existing guidelines or current screening practice, guidelines addressing only screening for moderate and/or high-risk population, older versions of an existing guideline, society guidelines that issue identical recommendations to multi-society or national guidelines or guidelines that were only published incomplete [*i.e.*, Australian Government NHMRC guidelines]. In case of an existing national guideline, more regional guidelines for that given country were excluded, as were guidelines or position papers addressing only one screening modality, guidelines or position papers providing only combined recommendations for average-risk and moderate/high-risk populations [*i.e.*, such as the Gastroenterological Society of Australia's (GESA) guidelines[16]], and publications in languages other than English. The British[17] and the New Zealand[18] guidelines were both excluded because they only issued recommendations for moderate to high-risk individuals and no specific guidelines for average risk individuals.

RESULTS

Included recommendations

The systematic database search yielded 1360 records and nine additional records were identified by hand searching. Overall, 1369 records were screened. From these, forty-six full texts were identified and screened further. Fifteen guidelines corresponding to the selection criteria were included in this systematic review (Figure 1).

Current guidelines follow as:

North America

Six guidelines were published in North America. A summary and their respective ratings of evidence are shown in Table 1.

American College of Gastroenterology (ACG, 2009): The ACG guidelines distinguishes prevention tests from detection tests[19]. Prevention tests, such as FS, colonoscopy and CT colonography, allow physicians to identify cancer and precursor lesions, whereas detection tests (fecal tests) have low sensitivity for adenomatous polyp detection and lower sensitivity than prevention tests for cancer. The preferred screening test recommended by ACG is colonoscopy, repeated every 10 years, starting at age 50 (strong recommendation, moderate-quality evidence) except for African Americans in whom screening should start at age 45 instead of age 50 (weak recommendation, low or very low-quality evidence). No upper age limit is recommended. However, if colonoscopy is not an option because of unavailability or individual preference, another prevention test, such as FS, repeated every 5-10 years (weak recommendation, moderate-quality evidence) or CT colonography, repeated every 5 years (strong recommendation, low or very low-quality evidence) is suggested. If the individual declines prevention tests, a detection test should be offered. The preferred detection test is annual FIT (strong recommendation, moderate-quality evidence), but alternatives are annual Hemoccult Sensa (gFOBT) (strong recommendation, moderate-quality evidence) or sDNA testing every 3 years (weak recommendation, moderate-quality evidence).

American College of Physicians (ACP, 2015): The ACP recommends screening for individuals between 50 to 75 years[20], using one of four suggested modalities: "high-sensitivity FOBT" or FIT (annually), FS every 5 years, colonoscopy every 10 years, or a combination of "high-sensitivity" FOBT/FIT (every 3 years) and FS (every 5 years). The ACP does not favor any one of these tests over another. According to this position statement, individuals 75 years or older and people with a life expectancy less than ten years should not undergo screening.

US Preventive Services Task Force (USPSTF, 2016): The USPSTF, an independent panel of experts, recommends screening average-risk individuals from age 50 to 75 (grade A recommendation)[13]. It is estimated that the benefit risk ratio decreases after age 75, especially in individuals with prior screening history. However, a healthy individual aged 76 to 85 without previous screening will likely benefit from screening[13]. For individuals between 76 to 85 years, screening is defined as a personal decision (grade C recommendation). No ranking was established among screening tests, since the USPSTF's goal is to maximize overall screening uptake, no matter which test is employed. It is mentioned that all screening tests have certain advantages and limitations and no one screening test has been identified to be superior to all others. Therefore, individuals undergoing screening should be allowed to choose their preferred screening option amongst the following options: annual high-sensitivity gFOBT, annual FIT, sDNA test every 1 to 3 years, FS every 5 years, colonoscopy every 10 years, CT colonography every 5 years, or a combination of FS every 10 years with annual FIT.

Canadian Task Force on Preventive Health Care (CTFPHC, 2016): The CTFPHC, comprised of an independent group of experts, recommends screening individuals aged 60 to 74, using gFOBT or FIT every two years, or FS every 10 years (strong recommendation,

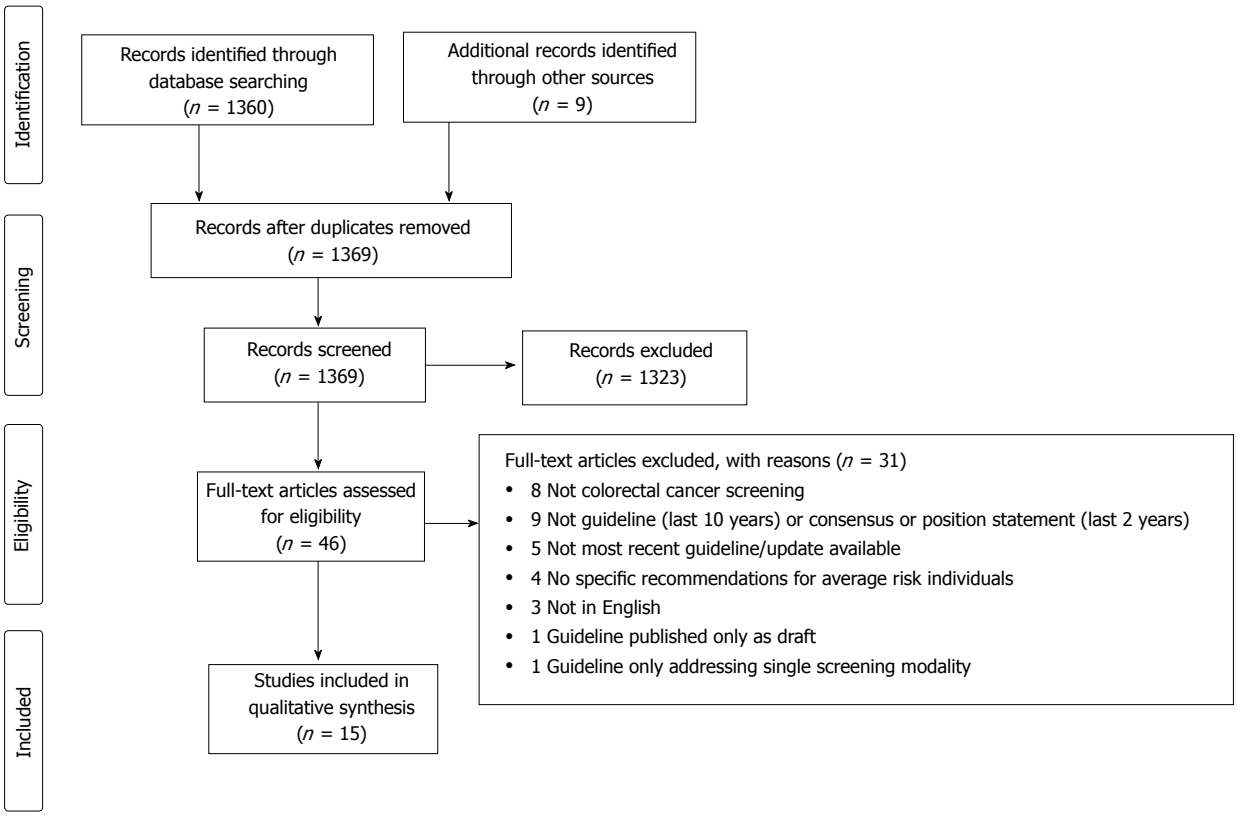

Figure 1 Prisma diagram.

moderate-quality evidence)[12]. Individuals aged 50 to 59 can get screened, using the same modalities (weak recommendation, moderate-quality evidence), however the benefit-harm ratio might be less favorable in this age group. According to CTFPHC, individuals between ages 50 and 59 can decide to defer screening until 60. Hence personal concerns and preferences should be discussed. Screening individuals beyond 75 is not recommended (weak recommendation, low-quality evidence), based on the absence of randomized controlled trials (RCTs) showing a reduction in CRC mortality and morbidity in this age group. The CTFPHC recommends against colonoscopy for screening (weak recommendation; low-quality evidence), based on the lack of high-quality evidence proving its efficacy when compared to other screening tests. Indeed, even if colonoscopy might provide benefits that are equivalent or greater to those obtained with FS, it requires a greater amount of resources and carries an increased risk of complications. However, if an individual prefers undergoing a colonoscopy, it can be considered[12].

National Comprehensive Cancer Network - (NCCN Guidelines, 2017): The working group suggests screening average-risk individuals starting at age 50. For individuals aged 76 to 85, screening is recommended as an individual decision, depending on overall health status and comorbidities in these individuals. Subjects in this age category who most likely benefit from screening are those who have not had a prior screening test. No preferred screening test is recommended, but different options are suggested, all are recognized as appropriate, however, some are based on high-level evidence, and identified as category 1, while others are recommended based on low-level evidence (category 2A; Table 1). Screening recommendations include colonoscopy every 10 years (category 2A), annual high sensitivity gFOBT (category 1) or FIT (category 2A), sDNA test every 3 years (category 2A), FS every 5 to 10 years (category 1), FS every 5 to 10 years combined with gFOBT/FIT at year 3 (category 2A), and CT colonography every 5 years (category 2A)[15]. These guidelines also mention that FIT is more sensitive than gFOBT.

United States Multi-Society Task Force of Colorectal Cancer Guidelines (2017): The working group of experts, representing the American College of Gastroenterology, the American Gastroenterological Association and the American Society for Gastrointestinal Endoscopy, recommends screening average-risk individuals starting at age 50 (strong recommendation; high-quality evidence), except for African Americans, in which screening should start at age 45 (weak recommendation; very-low-quality evidence)[21]. Screening should be interrupted at age 75 in individuals with negative prior screening or when life expectancy does not exceed 10 years (weak recommendation; low-

Table 1 Summarized recommendations for colorectal cancer screening in average-risk individuals, published in North America between 2007 and 2017

Continent	Country/association	Publication year	Age	Screening tests recommended	Recommendation	Note
North America	United States: ACG	2009	⩾ 50	Preferred prevention test: Colonoscopy (10 yr). If not possible or refused by individual: FS (5-10 yr) - OR CTC (5 yr) OR detection test	Grade 1B except for FS (2B) and CTC(1C)	Screening starting at age 45 for African American population
				Preferred detection test: FIT (1 yr). If not possible: Annual gFOBT (Hemoccult Sensa) OR- Fecal DNA testing (3 yr)	FIT : Grade 1B	
	United States: ACP	2015	50-75	High sensitivity FOBT/FIT (1 year) OR FS (5 years) OR FOBT/FIT (3 yr) + FS (5 yr) OR colonoscopy (10 yr)	-	
			⩾ 75 and individuals whose life expectancy is estimated to less than 10 years	Screening not recommended	-	
	United States: USPSTF	2016	50-75	gFOBT/FIT (1 yr) OR FIT-DNA (1-3 yr) OR FS (10 yr) + FIT (1year) OR FS (5 yr) OR colonoscopy (10 yr) OR CT-colonoscopy (5 yr)	Grade A recommendation	
			76-85	Screening is considered an individual decision,	Grade C recommendation	
	Canada: CTFPHC	2016	50-59	gFOBT/FIT (2 yr) OR FS (10 yr) OR defer until age 60	Weak recommendation; moderate-quality evidence	Colonoscopy not recommended for screening (weak recommendation; low-quality evidence), but could be discussed
			60-74	gFOBT/FIT (2 years) OR FS (10 yr)	Strong recommendation; moderate-quality evidence	
			⩾ 75	Screening not recommended, but can be discussed	Weak recommendation; low-quality evidence	
	United States: NCCN	2017	50-75	Colonoscopy (10 years) OR gFOBT/FIT (1 yr) OR Fecal DNA test (3 yr) OR FS (5-10 yr) (+/- gFOTB/FIT at year 3) OR CTC (5 yr)	Category 2A except for annual gFOBT and FS every 5-10 years (which are category 1)	FIT is identified as more sensitive than gFOBT
			76-85	Screening should be an individual decision, can be discussed		
	United States: US Multi-Society Task Force of Colorectal Cancer	2017	50-75	First-tier (preferred tests): Annual FIT OR colonoscopy (10 yr)	Strong recommendation; moderate-quality evidence	Screening for African American starting at age 45 (weak recommendation; very-low-quality evidence)
				Second-tier: CTC (5 yr) OR FIT-fecal DNA testing (3 yr) OR FS (5-10 yr)	CTC and FIT-DNA : Strong recommendation; low-quality evidence FS: Strong recommendation; high-quality evidence	
				Third-tier: Capsule colonoscopy (5 yr)	Weak recommendation; low-quality evidence	
			76-85	Screening should be considered for individuals without prior screening	Weak recommendation; low-quality evidence	

CRC: Colorectal cancer; FS: Flexible sigmoidoscopy; DCBE: Double contrast barium enema; CTC: CT colonography, FOBT: Fecal occult blood test: gFOBT: Guaiac-based fecal occult blood test: FIT: Fecal immunochemical test.

Table 2 Summarized recommendations for colorectal cancer screening in average-risk individuals, published in Europe between 2007 and 2017

Continent	Country/Association	Year	Age	Screening tests recommended	Recommendation	Note
Europe	Scotland: TIS	2011 (revised in 2016)	Age not mentioned	FIT (quantitative) (interval not mentioned)	Grade A recommendation	Performance of FS unsure in the Scottish population. Colonoscopy and CT colonography are not recommended
	Germany: GGPO	2014	≥ 50	Preferred test: Colonoscopy (10 yr) If refused by individual: FS (5 yr) + annual FOBT OR Annual FOBT	Colonoscopy: Grade B recommendation; 3b level of evidence. FS: Grade B recommendation; 2b level of evidence. Adding FOBT to FS: Grade B recommendation; 3b level of evidence. FOBT as a screening test: Good clinical practice	General use of FIT is not recommended, but FIT can be used instead of gFOBT if it has a proven high specificity (> 90%) and sensitivity. Genetic stool tests, CT colonography, MR-colonography and capsule endoscopy are not recommended.
	Spain: SEOM	2014	50-74	FIT every 2 yr OR, depending on available resources, annual or biennial gFOBT OR FS (5 yr) OR colonoscopy (every 10 yr)	Grade B (moderate) quality of evidence, except for FOBT every 2 yr (grade A quality of evidence)	Combination of gFOBT and FS, and CT colonography are not recommended
	European Guidelines	2013	50-74	Recommended test: gFOBT/FIT (1-2 yr) Other options include colonoscopy (10-20 yr) OR FS (10-20 yr)	Recommendation based on good evidence for gFOBT, reasonable evidence for FIT and FS, and limited evidence for colonoscopy	Evidence supports FIT superiority compared to gFOBT

CRC: Colorectal cancer; FS: Flexible sigmoidoscopy; FOBT: Fecal occult blood test; gFOBT: Guaiac-based fecal occult blood test; FIT: Fecal immunochemical test.

quality evidence). However, individuals without prior screening could still benefit from screening and therefore could undergo screening until age 85, depending on their age and comorbidities (weak recommendation; low-quality evidence). The panel ranked screening tests in 3 tiers based on performance, costs and practical considerations. Colonoscopy every 10 years and annual FIT are ranked as first-tier and therefore are recommended as preferred tests (strong recommendation; moderate-quality evidence). CT colonography every 5 years, FIT-DNA test every 3 years (both strong recommendation; low-quality evidence) and FS every 5 to 10 years (strong recommendation; high-quality evidence) are ranked as second-tier tests. Capsule colonoscopy every 5 years, however, is ranked as a third-tier test (weak recommendation; low-quality evidence)[21].

Europe

Four different European guidelines were included in this review. A summary of recommendations and adopted methodology for rating of evidence are shown in Table 2.

European Colorectal Cancer Screening Guidelines Working Group (2013): The European Colorectal Cancer Screening Guidelines Working Group (working group of experts) recommends screening individuals between ages 50 and 74[22,23]. FOBT is mentioned as the only screening method approved throughout the European Union (EU). The guideline mainly provides information on how to set up screening programs of great quality, using the most commonly used modalities in Europe, which are FOBT, FS and colonoscopy[22,23]. As for stool-based tests, gFOBT and FIT are recognized as effective, but it is suggested that quantitative FIT is superior in terms of specificity and sensitivity and is recommended over gFOBT. FOBTs should be repeated on an annual or biennial basis or, at the very least every three years if FIT is used[22]. The guidelines highlight the lack of high quality evidence assessing colonoscopy. However, according to the authors, current evidence support 10 year surveillance if colonoscopy is used, suggesting that interval extension to 20 years might be appropriate[22]. FS is discussed as potential screening test, but no screening interval is clearly defined; the authors suggest using the same interval as for colonoscopy screening[22]. FOBT with FS, CT colonography, stool DNA testing and capsule endoscopy are not recommended[22].

German Guideline Program in Oncology (2014): The German Guideline Program in Oncology (GGPO), a working group of experts, recommends screening starting at age 50[24]. The GGPO does not establish an upper age screening limit, citing a lack of studies concerning benefit to risk ratio in older individuals. The decision

should be based on a subject's health with associated comorbidities. Here too, the guideline distinguishes cancer prevention (colonoscopy, sigmoidoscopy, CT-colonography, capsule endoscopy) from cancer detection (FOBT, genetic stool tests) tests. Colonoscopy is recommended as gold standard, and should be repeated every 10 years (grade B recommendation; 3b level of evidence). Based on indirect evidence, colonoscopy is recommended as the most specific and sensitive screening test for the detection of cancer and adenomas. If an individual refuses colonoscopy, FS should be offered every 5 years (grade B recommendation; 2b level of evidence), combined to an annual FOBT for assessment of the proximal colon (grade B recommendation; 3b level of evidence). Because a positive FOBT needs to be followed up with a complete colonoscopy, any annual FOBT should be completed before the associated FS in order to avoid unnecessary FS. FOBT alone is recognized as an effective screening test, and should be repeated annually rather than once every two years (1b level of evidence) in individuals refusing colonoscopy (this recommendation is identified as good clinical practice). There exist a variety of FIT modalities offered in Germany with greatly varying specificities and sensitivities, making it difficult to favor FIT as a blanket statement over gFOBT. However, a given FIT test could replace gFOBT if its given specificity has been shown to be greater than 90%, while also exhibiting a high sensitivity (grade 0 recommendation; 3a level of evidence). Genetic stool tests were not recommended for CRC screening, because of insufficient data (grade B recommendation; respectively 3b and 4 levels of evidence). Radiologic screening modalities such as CT- and MR-colonography were not recommended, but could be used in case of an incomplete colonoscopy in an individual requesting a complete colon examination (grade B recommendation; 3b level of evidence)[24].

Spanish Society of Medical Oncology (SEOM, 2014): The Spanish Society of Medical Oncology recommends screening for average-risk individuals between ages 50 and 74. Biennial FOBT is recommended based on high-quality evidence (grade A) with FIT considered as the preferred test. As alternative to FIT, annual or biennial high-sensitivity gFOBT, FS repeated every 5 years or colonoscopy repeated every 10 years can be used (grade B quality of evidence). Based on moderate-quality evidence (grade B), the SEOM recommends against using a combination of FS and gFOBT. It also recommends against the use of CT colonography until sufficient data become available (grade B quality of evidence)[25].

Scottish Intercollegiate Guidelines Network (Healthcare Improvement Scotland, 2016): According to the Scottish Intercollegiate Guidelines Network the most appropriate tool for population screening is a quantitative FIT (grade A recommendation). Although no specific fecal hemoglobin concentration cut-off is identified, the working group suggests using a cut-off value that is higher than the sensitivity of gFOBT. The guidelines state FS has been proven to be an efficacious screening test, perhaps more so than FIT, but its effectiveness is unproven if offered to the Scottish population and it is therefore not recommended; neither are colonoscopy nor CT colonography[14]. The guideline does not specify an age range nor surveillance intervals following a negative FIT.

Asia

Four different Asian guidelines were included in this systematic review. Guideline recommendations from Asia and methodology for rating of evidence are summarized in Table 3.

Korean Guidelines for Colorectal Cancer Screening and Polyp Detection (2012): The Korean Multi-Society Task Force recommends screening for average-risk individuals starting at age 50 (strong recommendation; low-quality evidence)[26]. No upper age limit is identified. Colonoscopy is the preferred screening test (strong recommendation; low-quality evidence), and should be repeated every 5 years (weak recommendation; very low-quality evidence). FOBT is another recommended option (strong recommendation; moderate-quality evidence), but FIT should be used rather than gFOBT because of higher specificity, convenience and compliance (strong recommendation; low-quality evidence). Other screening tests such as CT colonography (strong recommendation; low-quality evidence) and DCBE (weak recommendation; low-quality evidence) are also identified as possible options. The efficacy of FS is recognized, and FS is listed as a potential screening test, but the consensus document states this modality is not commonly employed in Korea since it does not investigate the entire colon, and must be followed by a colonoscopy if positive; the guideline also states that, in Korea, individuals and physicians often prefer colonoscopy[26].

Chinese Society of Gastroenterology (2014): Given its large national population and attendant resources utilization issues, the Chinese Society of Gastroenterology consensus does not recommend colonoscopy or FS as first line screening test for average-risk individuals. The guidelines suggest that individuals between ages 50 and 74 undergo FOBT and that a questionnaire be used to identify high-risk factors. The immunoassay FOBT should be preferred over a chemical FOBT, however, the guidelines also suggest gFOBT followed by FIT can be used. Individuals are should undergo colonoscopy if they meet any one of the five following conditions: (1) positive FOBT; (2) history of CRC in first-degree relatives; (3) personal history of intestinal adenomas; (4) personal history

Table 3 Summarized recommendations for colorectal cancer screening in average risk individuals, published in Asia between 2007 and 2017

Continent	Country/region	Year	Age	Screening tests recommended	Recommendation	Note
Asia	South Korea	2012	⩾ 50	Colonoscopy (at least 5 years) is the priority OR FOBT (FIT) OR CTC OR DCBE	Colonoscopy (strong recommendation; low-quality evidence) with 5-year interval (weak recommendation; very low-quality evidence). FOBT (strong recommendation; moderate-quality evidence). CTC (strong recommendation; low-quality evidence). DCBE (weak recommendation; low-quality evidence)	FS efficacy is recognized, but FS not widely used because it doesn't explore entire colon, might need a colonoscopy after, and FS less preferred by individuals and physicians
	China	2014	50-74	FOBT (chemical FOBT or FIT) + Questionnaire every 3 yr	-	
	Asia Pacific	2015	50-75	FIT (preferred choice) OR FS OR colonoscopy (intervals not mentioned)	A for FIT; A for FS; B for colonoscopy	FIT is preferred over gFOBT
	Saudi Arabia	2015	45-69	Colonoscopy (10 yr) is the recommended modality; if not possible: FS (5 yr)+ FIT/gFOBT (1 yr) OR FS (3 yr)	Colonoscopy: Strong recommendation; low-quality evidence. FS: Strong recommendation; moderate-quality evidence.	FIT is preferred over gFOBT. FOBT used alone is not recommended, but could be used depending on availability of other modalities.
			⩾ 70	Screening not recommended	Conditional recommendation; low-quality evidence	Screening for people over 70 could be beneficial in certain cases (depending on health status)

CRC: Colorectal cancer; FS: Flexible sigmoidoscopy; DCBE: Double contrast barium enema; CTC: CT colonography; FOBT: Fecal occult blood test; gFOBT: Guaiac-based fecal occult blood test; FIT: Fecal immunochemical test.

of cancer; and (5) or if meeting two of the six following criteria: history of chronic diarrhea, chronic constipation, bloody mucus, chronic appendicitis or appendectomy, chronic cholecystitis or cholecystectomy, or a long-term mental depression. If colonoscopy is not available FS is recommended. Screening should be repeated every 3 years[27].

The updated Asia Pacific Consensus Recommendations on colorectal cancer screening (2015): The Asia Pacific Working Group recommends screening average-risk individuals between 50 and 75 (grade B recommendation; II-2 quality of evidence)[28]. Utilization of a stool-based test is recommended (grade A recommendation; I quality of evidence). Quantitative FIT should be used over gFOBT (grade A recommendation; I quality of evidence), because of its higher sensitivity, specificity and individual adherence. FS is considered appropriate for screening (grade A recommendation; I quality of evidence), as is colonoscopy (grade B recommendation; II-2 quality of evidence). Colonoscopy is considered the gold standard among endoscopic modalities. However, considering resource-limitations for population based screening, the consensus recommendations state that using FIT is the preferred choice for average risk screening and colonoscopy resources should be used for screening of high risk individuals. With regards to surveillance intervals, the guidelines review the literature supporting 1-2 years for FIT, and 10 years for colonoscopy based on previous studies or other guidelines, however, the guideline itself does not provide specific recommendations. CT colonography and capsule endoscopy are not recommended but mentioned as appropriate for individuals in whom colonoscopy is not possible (grade B recommendation; II-1 and II-2 quality of evidence, respectively)[28].

National Guidelines for Colorectal Cancer Screening in Saudi Arabia (2015): The national Saudi guidelines published by a working group of experts recommend screening for CRC in average-risk individuals starting at age 45, based on a median national age of CRC diagnosis of 55 for women and 60 for men[29]. Screening individuals aged 70 or more is not recommended (conditional recommendation; low-quality evidence) because of the risks of complications. However, it is mentioned that certain individuals could benefit from screening after age 70, if they have no comorbidities and an expected life expectancy of 10 years or more. The preferred modality in this guideline is colonoscopy, repeated every 10 years (strong recommendation; low-quality evidence). Use of colonoscopy is preferred over FS (conditional recommendation; low-quality evidence), since it examines the full colon, and has to be less frequently repeated. However, the guidelines also recommend FS, repeated

Table 4 Recommended test in terms of available resources according to World Gastroenterology Organization's colorectal cancer screening cascade

Level of recommendation	Recommended screening test
1	Colonoscopy every 10 yr
2	Colonoscopy, once in a lifetime
3	FS every 5 yr, followed by a colonoscopy if FS was positive
4	FS, once in a lifetime, followed by a colonoscopy if FS was positive
5	FS, once in a lifetime, followed by a colonoscopy only if advanced neoplasia is detected
6	Fecal blood test annually, followed, if positive, by a colonoscopy or barium enema (depending on colonoscopy's availability)

FS: Flexible sigmoidoscopy.

every 3 years, as alternative (strong recommendation; moderate-quality evidence). This test is considered more feasible than colonoscopy, but less favored in Saudi Arabia. FS is preferred over gFOBT for screening average-risk individuals (conditional recommendation; very low-quality evidence). This guideline does not recommend stool-based tests if used alone, but these can be offered depending on the availability of other modalities. Nonetheless, the possibility of combining an annual stool-based test with FS, repeated every 5 years, is recommended to maximize screening benefits. The superiority of FIT over gFOBT is also mentioned[29].

World Gastroenterology Organization (WGO, 2007)

The WGO issued a CRC screening cascade with recommendations based on resource availability. Six different levels, ranging from 1 (best resource availability) to 6 (minimal resource availability), are detailed. All recommendations apply to average-risk individuals 50 years or older (Table 4). No upper age limit is identified. CT colonography and DNA testing are not included in the cascade, but they are mentioned as alternate modalities if an individual refuses to undergo other recommended tests[30].

DISCUSSION

The vast majority of guidelines recommend starting CRC screening for average-risk individuals at age 50. This is based on the steep increase of CRC beginning around age 50. In 2009, 90% of worldwide CRC were diagnosed in individuals aged 50 or more[31]. A comparative effectiveness modeling completed by the USPSTF showed that starting screening at age 45 instead of 50 in average-risk population could result in a modest increase of life-years gained, but also in an increase in the lifetime number of colonoscopies, worsening the burden of screening for individuals[13]. The CTFPHC guidelines (Canada) suggest starting screening at age 50, while allowing to defer screening until age 60[12]. Several European programs start screening around age 60, which is justified by the higher prevalence of CRC after this age[32,33]. In fact, the majority of CRC cases in United States are diagnosed between 65 to 74 years[34]. However, African Americans have a higher prevalence of CRC and consequently the ACG recommends screening for African American individuals to start at age 45[35]. Interestingly, Saudi Arabia, also recommends starting to screen at age 45 because the median age at time of CRC diagnosis is 55 in Saudi women and 60 in Saudi men[29], as compared to 70 in Canada[36] and 68 in United States[34].

Ten of fifteen guidelines identified recommend an upper age screening threshold varying from age 70 to 75, based on associated harms potentially exceeding benefits if screening is continued after that point[37]. Nonetheless, as screening might still be beneficial in selected elderly individuals, the decision to stop screening should be individualized[12,13,29]. The pertinence of setting 75 as the maximal screening age, instead of a higher threshold fixed at 85 years old, has been demonstrated by Zauber *et al*[38] in 2008. The study showed that reducing the upper age limit from 85 to 75 leads to small decreases in life-years gained, but also results in a great reduction of colonoscopy use, making age 75 likely to be more beneficial in a population based screening environment.

As for screening modalities, all guidelines have considered gFOBT, FIT, FS and colonoscopy as mainstays of CRC screening. However, there exist discrepancies with regards to which test(s) should be preferred. FOBTs are widely used, being recommended either as preferred test or not based on whether the context is that of population-based screening or an area with limited endoscopy resources. Even though RCTs have clearly demonstrated the efficacy of gFOBT with such evidence lacking for FIT, several guidelines suggest FIT is superior to gFOBT because of its greater specificity and sensitivity[39]. FIT is also associated with improved adherence[40], and does not require dietary restrictions. Stool-based tests are recommended on an annual or biennial basis[9]. As annual FOBT has been shown to decrease CRC-related mortality[8] and increase the number of life-years gained compared to biennial FOBT[38], the majority of guidelines suggest 1-2 year intervals for FOBT screening. Optimal diagnostic FIT threshold levels of positivity remain an area of uncertainty that has not been directly discussed with guideline recommendations.

Major disparities throughout the different guidelines

can be found relating to the use of endoscopy. While colonoscopy is often referred to as the gold standard, and is suggested as preferred screening test by many guidelines, others recommend FS based on available higher quality evidence. This area of controversy is best illustrated by the CTFPHC recommendations (Canada) on CRC screening. Authors conclude that the available evidence supports using guaiac fecal occult blood testing (gFOBT) and flexible sigmoidoscopy for CRC screening because these modalities have been shown to reduce mortality while such evidence does not exist for colonoscopy, and therefore recommend against using colonoscopy as a screening test. This recommendation is graded as a weak one, which means that a majority of people would not want colonoscopy, but many would[12]. It is interesting to notice that current literature was interpreted differently by other guidelines, such as USPSTF's, which strongly recommended colonoscopy, based on moderate-quality evidence[21]. What is even more interesting is that both CTFPHC and USPSTF used the Grading of Recommendations Assessment, Development and Evaluation (GRADE) system, but ended up drawing different conclusions[12,21]. This might be explained by USPSTF's more flexible approach: the working group used a modified qualitative approach based on a review of the literature, but did not include a meta-analysis (which is usually included in GRADE system)[21]. In CTFPHC's case, using a rigid approach led to recommending FS over colonoscopy, but it is unlikely that such a recommendation would change current screening practice, which include colonoscopies on a regular basis, even if high-quality evidence is not currently available. Appropriate colonoscopy studies addressing this lack have been initiated and should be completed between 2021 and 2036[41-46]. In the meantime, other guidelines recommend colonoscopy based on case-control and prospective cohort studies that suggest it results in a reduction in CRC-related mortality ranging from 65% to 88%[7,47-49]. The screening interval following a negative colonoscopy is usually set at 10 years, based on the natural history of progression of adenomas into carcinomas[50,51]. In the case of FS, suggested screening intervals vary from 3 to 10 years; more evidence is required to determine optimal screening intervals, especially after colonoscopy and RCTs addressing this important area of uncertainty are underway[52]. Guidelines published by the ACP, NCCN, USPSTF, Saudi Arabia and GGPO all suggest the possibility of combining FOBT and FS[13,15,20,24,29]. Adding FIT to FS increases sensitivity for detecting proximal invasive cancer, while also providing a 10% increase in higher sensitivity for advanced distal neoplasia. Combining both tests generates better results than using either test alone[53]. Screening intervals for such combination have not been established, but combining the intervals used for FS (5 years) with an additional FIT every 1-2 years seems reasonable.

Individual's adherence to a screening modality is an important factor when it comes to efficient CRC screening, hence the importance to select a test that makes it easy for a patient to adhere. Less invasive procedures are usually more accepted by individuals than more invasive procedures, and therefore, higher participation rates can be noted. Studies have shown that higher adherence rates were obtained with gFOBT, FIT[54] and FS when compared to colonoscopy[55] and CT colonography[56] (see Table 5). There is evidence that FIT is more accepted than gFOBT because it only requires one stool sample and no dietary restrictions[40,57]. Participation rates for FS were equal to participation rates for FIT in a study[55], while they were lower than the latter according to another[56]. An article published in 2012 documented that the most frequently cited reason to decline colonoscopy was unpleasantness of the examination, whilst the most frequent reasons to decline CT colonoscopy were ‘’ no time/too much effort’’ and lack of symptoms[58]. Less invasive and less time-consuming procedures such as gFOBT and FIT could therefore be more easily accepted by individuals.

When it comes to cost-effectiveness, gFOBT, FIT, FS, colonoscopy, sDNA and CT colonography are all cost-effective in comparison to no screening[59]. Prices differ between tests, gFOBT and FIT being the two most affordable ones, with costs ranging from 5 to 23 USD and 23 to 25 USD, respectively[60,61] (see Table 5). However, a lower cost per test is not necessarily associated with higher cost-effectiveness. Even though colonoscopy is currently one of the most expensive screening test available, Patel and Kilgore showed that colonoscopy every 10 years was cost-effective when compared to annual FOBT or FS every 5 years. A combination of FS every 5 years and annual FOBT was also better than either test alone[59].

All recommendations considered, there appears to be no single "best" CRC screening test for an average risk individual. The preferred modalities include FOBT, FS or colonoscopy and the appropriate choice should be based on local resource availability and individual willingness to undergo and adhere to the chosen test and surveillance requirements. The WGO created a screening cascade with six levels of recommendations, graded according to available resources (Table 4)[30]. The first level constitutes the ‘’best-case scenario’’ (if all resources are available), while the last one would be the ‘’worst-case scenario’’ (with very limited resources). The USPSTF also ranked screening methods in three tiers, depending on performance, costs and practical considerations[21]. Such ranking is useful in clinical practice compared to a menu of options where no clear indication is given about which test should be prioritized. A screening cascade or ranking can therefore guide the physician while allowing a certain flexibility when it comes to choosing a screening test. Guidelines from USPSTF and CTFPHC[12,13], emphasize

Table 5 Screening tests characteristics

Screening test	Specificity/sensitivity for advanced adenoma detection (%)	Specificity/sensitivity for CRC detection (%)	Price (USD)	Participation rates after first-time invitation (%)[56]	Decreased mortality for CRC (%)	Risk of complications (%)[63]
gFOBT	95.4/8.6[64]	97.7/23.8[59]	5[61]-23[60]	47	14[65]-32[7]	0
FIT	96.8-97.4/20.3-25.7[64]	94.0 79.0[66]	23[60]-25[61]	42	59[65]	0
FS	87.0/95.0[67]		169[60]-238[61]	35	33[65] - 50[10]	Perforation: 0.01 Major bleeds: 0.02
Colonoscopy	91.3/92.9 (for adenomas > 10 mm)[68]	100.0/91.2[68]	645[60]-803[61]	28	61[65]- 65[48]	Perforation: 0.04 Major bleeds: 0.08
sDNA test	89.8[1]/42.4[69]	89.8[1]/92.3[69]	150[61]	NR	NR	0
CTC	87.3/91.2 (for adenomas > 10 mm)[68]	99.0/96.8[68]	570[60]	22	NR	Perforation: Less than 0.02

[1]Specificity was not defined separately for advanced adenoma detection and colorectal cancer detection. CRC: Colorectal cancer; gFOBT: Guaiac fecal occult blood test; FIT: Fecal immunochemical test; FS: Flexible sigmoidoscopy; sDNA test: Stool DNA test; CTC: CT colonography; NR: Not reported.

that individual screening preferences should be considered to optimize screening uptake[62] - *i.e.:* any screening test is better than none.

Only guidelines published in English were included, resulting in an over-representation of North American recommendations compared to other continents, and potentially limiting the generalizability for any true global overview. Interestingly, there are also a number of English speaking countries that are not represented in this review because their guidelines did not include recommendations for average-risk individuals (such as the British[17] and the New Zealand[18] guidelines) or issued only combined recommendations for average and moderate-risk individuals (such as the Gastroenterological Society of Australia's guidelines[16]) or are at an incomplete publication stage. *i.e.*, with the Australian Government NHMRC guidelines being currently under revision and not published as final version, no Australian guideline has been included in our review. It is also important to note that many of the reviewed guidelines adopted their own system to grade the strength of recommendation/evidence, limiting a more direct comparison.

In conclusion, average-risk individuals aged 50 to 75 should undergo CRC screening. Screening for individuals below 50 and above 75 should be individualized, but it is important to consider stopping screening at a certain age. Colonoscopy (every 10 years), FS (every 5 years) and annual or biennial FOBT are the most common recommended modalities for CRC screening. The superiority of FIT when compared to gFOBT has been established with regards to test performance characteristics, while a combination of FIT and FS has been associated with better results than either test alone. Despite the current absence of RCT data, colonoscopy is considered the preferred screening modality in many CRC guidelines. Ideal screening intervals remain an area of uncertainty and is currently under investigation in RCTs. Finally, resource availability and individual preferences should be considered when choosing the most appropriate screening intervention to improve the uptake of and optimize the real-life effectiveness of CRC screening.

ARTICLE HIGHLIGHTS

Research background

Screening has shown to decrease colorectal cancer (CRC) incidence and mortality. Different screening guidelines for average-risk individuals have been issued worldwide, and several guidelines were published or updated recently. To our knowledge, this is the first systematic review aiming to summarize and compare worldwide CRC screening recommendations.

Research motivation

CRC screening recommendations for average-risk individuals differ greatly from one guideline to another, especially when it comes to choosing a preferred screening test. We aimed to compare those recommendations in order to highlight areas of uncertainty, and therefore orient future research by underlining areas where evidence is still lacking.

Research objectives

The main objectives were to compare screening recommendations in order to highlight common ground between guidelines, but also point out discrepancies caused by lack of high-quality evidence, making it easier to orient future research.

Knowing which recommendations should clearly be perpetuated and which ones need further investigation can be helpful when it comes to updating guidelines or publishing new ones.

Research methods

A systematic review of the literature was completed to identify all CRC screening guidelines for average-risk individuals published in English in the last ten years and/or position statements published in the last two years. Articles describing an established screening program without issuing recommendations, or articles only reviewing existing guidelines were excluded. Guidelines providing combined recommendations for average-risk and moderate/high-risk individuals, addressing only screening for moderate/high-risk individuals or older versions of existing guidelines were also excluded.

Research results

Fifteen guidelines were included, six of which were published in North America, four in Europe, four in Asia and one by the World Gastroenterology Organization (WGO). A majority of guidelines recommend screening average-risk individuals between ages 50 and 75. Preferred screening methods include colonoscopy (every 10 years), flexible sigmoidoscopy (FS - every 5 years), guaiac-based fecal occult blood test (gFOBT) or fecal immunochemical test (FIT), both repeated annually or biennially. FIT is often recommended over gFOBT, and combining FS with a stool based test is an option that should be considered. The role of colonoscopy varies greatly from one guideline to another, as some identify it as the screening gold standard whilst others highlight the lack of high-quality evidence supporting its use. Screening intervals as well as rank order between tests are also areas of uncertainty.

Research conclusions

Average-risk individuals should undergo CRC screening between ages 50 and 75. Colonoscopy, FS, gFOBT and FIT are recognized as cost-efficient and currently recommended in a majority of guidelines, however their respective role and rank are not clearly established. Local resources availability and patient preferences should be considered when implementing a screening program, in order to maximize screening uptake, as any screening is better than none.

Research perspectives

Establishing a clear ranking of screening methods rather than simply offering a menu of options could be useful in clinical practice. Future research should aim to provide high-quality evidence demonstrating screening tests efficiency, especially colonoscopy, in order to facilitate comparison between tests and help establishing such ranking. Screening intervals should be further investigated.

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META-ANALYSIS

Probiotic monotherapy and *Helicobacter pylori* eradication: A systematic review with pooled-data analysis

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Abstract

AIM

To define probiotic monotherapy effect on *Helicobacter pylori* (*H. pylori*) status by performing a systematic review.

METHODS

Methods of analysis and inclusion criteria were based on PRISMA recommendations. Relevant publications were identified by searching PubMed, MEDLINE, Science Direct, and EMBASE. The end-point was to estimate eradication rate and urea breath test delta value before and after probiotic monotherapy across all studies and, overall, with a pooled data analysis. Adverse events of probiotic therapy were evaluated. The data were expressed as proportions/percentages, and 95%CIs were calculated. For continuous variables, we evaluated the weighted mean difference. Odd ratios (ORs) were calculated according to the Peto method for the comparison of eradication rates between probiotics and placebo.

RESULTS

Eleven studies were selected. Probiotics eradicated *H. pylori* in 50 out of 403 cases. The mean weighted eradication rate was 14% (95%CI: 2%-25%, *P* =

0.02). Lactobacilli eradicated the bacterium in 30 out of 235 patients, with a mean weighted rate of 16% (95%CI: 1%-31%). *Saccharomyces boulardii* achieved eradication in 6 out of 63 patients, with a pooled eradication rate of 12% (95%CI: 0%-29%). Multistrain combinations were effective in 14 out of 105 patients, with a pooled eradication rate of 14% (95%CI: 0%-43%). In the comparison of probiotics *vs* placebo, we found an OR of 7.91 in favor of probiotics (95%CI: 2.97-21.05, $P < 0.001$). Probiotics induced a mean reduction in delta values higher than placebo (8.61% with a 95%CI: 5.88-11.34, *vs* 0.19% for placebo, $P < 0.001$). Finally, no significant difference in adverse events was found between probiotics and placebo (OR = 1, 95%CI: 0.06-18.08).

CONCLUSION

Probiotics alone show a minimal effect on *H. pylori* clearance, thus suggesting a likely direct role.

Key words: *Helicobacter pylori*; Probiotics; Eradication; Meta-analysis; Breath test

Core tip: Despite several lines of evidence in the literature having demonstrated a pivotal role of probiotics as adjunctive treatment for *Helicobacter pylori* (*H. pylori*) eradication, national and international guidelines do not have a uniform consensus about their clinical application. Many meta-analyses have confirmed that co-administration of probiotics may have a beneficial effect on the prevention of side effects and eradication rates. Herein, we found that probiotic monotherapy may eradicate *H. pylori* in 14% of cases. Lactobacilli, *Saccharomyces boulardii* and multistrain combinations eradicated the bacterium with a rate of 16%, 12% and 14%, respectively. Probiotics were significantly more effective than placebo (OR = 7.91).

Losurdo G, Cubisino R, Barone M, Principi M, Leandro G, Ierardi E, Di Leo A. Probiotic monotherapy and *Helicobacter pylori* eradication: A systematic review with pooled-data analysis. *World J Gastroenterol* 2018; 24(1): 139-149 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i1/139.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i1.139

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a ubiquitous Gram-negative, flagellated organism, residing in the human stomach, where it may cause both malignant and nonmalignant diseases[1-3]. The treatment of *H. pylori* relies mainly on a combination of antibiotics. However, despite several therapeutic schemes having been proposed, the way towards ideal therapeutic management remains an unsolved issue[4].

Until a few years ago, triple therapy (based on a proton pump inhibitor, amoxicillin and clarithromycin) was considered as the standard first-line regimen. However, failure rates have increased recently, due to the spreading of antibiotic resistances, which are due to point mutations of the *H. pylori* genome[5]. For this reason, alternative first-line regimens have been proposed (sequential, concomitant, quadruple with and without bismuth, and hybrid). In this context, the geographic pattern of antibiotic resistances must also be studied as a relevant matter[6-9]. To now, the "ideal therapy" does not exist and this is the real limit for worldwide effective therapeutic guidelines[6].

A relevant problem related to *H. pylori* therapy failure is linked to patient compliance, which is often affected by antibiotic-associated adverse events, including diarrhea, nausea, vomiting and abdominal pain. Therefore, the development of a new strategy which could improve the eradication rate as well as reduce the frequency of adverse effects is advisable.

Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host"[10]. The intestinal microbiota is the community of microorganisms which colonizes the gut. It is an essential component of the luminal intestinal environment. Antibiotic-induced alteration of the microbiota may lead to diarrhea and other side effects[11]. Consequently, probiotic supplementation could restore microbial balance, thus preventing antibiotic-associated adverse events[12,13]. In particular, this benefit may be useful in *H. pylori* management for the need to administer a combination of antibiotics at high dose.

Furthermore, it is supposed that probiotics could interfere with potential pathogens which may colonize the stomach[14]. Indeed, probiotics may compete with *H. pylori* for host surface receptors and, thereby, inhibit its adhesion to epithelial cells[15]. Furthermore, it has been demonstrated that, *L. acidophilus* may hamper *H. pylori* urease activity *in vitro*[16]. Finally, lactobacilli produce lactic acid, which is able to counteract *H. pylori*-induced hypochlorhydria and has bactericidal effect itself[17]. For these reasons, it is possible to hypothesize that probiotics may exert a direct inhibitory effect on *H. pylori* growth.

Several meta-analyses have demonstrated that probiotics, when given in combination with the standard therapy, induce an improvement in both eradication rates and reduction of adverse events. In this regard, Zhang *et al*[18] demonstrated that probiotic administration along with triple therapy achieved a success rate of 82.31% (against the 72.08% of the control group), with a risk ratio of 1.11 in favor of probiotics. Another study[19] showed that probiotics have a positive effect on preventing diarrhea [odds ratio (OR) = 0.21] and increase the eradication rate, with an OR of 1.68.

Until now, meta-analyses have investigated pro-

biotic effects on *H. pylori* only in association with antibiotics. To the best of our knowledge, there are no meta-analyses concerning probiotic monotherapy effects on *H. pylori* infection. Therefore, our aim was to perform a systematic review with pooled data analysis regarding this uninvestigated topic.

MATERIALS AND METHODS

Eligibility criteria and study selection

Methods of analysis and inclusion criteria were based on "Preferred Reporting Items for Systematic reviews and Meta-Analyses" (PRISMA) recommendations[20], and the Meta-analysis Of Observational Studies in Epidemiology (MOOSE) checklist has been enclosed as supplementary material. We excluded review articles, experimental *in vitro* studies and single case reports.

Data collection process

A literature search was performed in May 2017. Relevant publications were identified by a search of PubMed, MEDLINE, Science Direct and Scopus. The search terms were *Helicobacter pylori*, probiotics, lactobacilli, bifidobacteria, saccharomyces, treatment, eradication, breath test. We used the following string, with Boolean operators AND/OR: ([Helicobacter pylori OR H. pylori] AND [probiotic* OR lactobacil* OR bifidobacteria OR saccharomyces OR bacillus OR treatment OR eradication OR breath test]). We excluded studies that used probiotics in combination with antibiotics, while co-administration of other molecules, such as proton pump inhibitors, was not considered as an exclusion criterion. We excluded, as well, studies in which patients with major gastrointestinal surgery interventions were enrolled.

Titles and abstracts of papers were screened by two reviewers (Losurdo G and Ierardi E). Studies were independently prescreened in blinded fashion for relevance by the two reviewers using full reports. Discussion put an end to any disagreements. Successively, data were extracted from the relevant studies by one reviewer and checked by a second reviewer, and thus inserted into dedicated tables. A third reviewer (Leandro G) came to a decision on any disagreements.

Reviewers independently extracted the following data from each paper: (1) year of publication; (2) country where the study was performed; (3) single- or multicenter study; (4) study design; (5) number of patients included; (6) mean age and sex of enrolled patients; (7) test used to diagnose *H. pylori* infection; (8) type of probiotic and modality of administration; (9) success rate; (10) delta values of urea breath test (UBT); and (11) adverse events. We did not include studies reporting only the results of UBT delta value without detailing eradication rate.

Summary measures and planned methods of analysis

The end-point was to estimate the mean eradication rate and variations of delta value at UBT across all studies and, overall, with a pooled data analysis. The data were expressed as proportions/percentages, and 95%CIs were calculated using the generic inverse variance method, as described in the Cochrane Handbook, Chapter 9.4.3.2[21], and as we already performed in a previous meta-analysis[22]. The inverse variance methods allow a "weighting" of the eradication rate according to the sample size. For continuous variables (delta value of UBT), we entered mean, standard deviations and sample size in order to calculate the weighted mean difference. OR and 95%CI were calculated, where available, based on the Peto method, for the comparison of two groups (probiotics *vs* placebo).

Data were entered into the RevMan 5.3 software (The Nordic Cochrane Centre, Copenhagen, Denmark) (Cochrane library) in order to draw forest plots. A P value < 0.05 was considered statistically significant. Heterogeneity was assessed by using the χ^2 and I^2 statistics. In particular, heterogeneity was considered to be present if the χ^2 test delivered a $P < 0.05$ and, therefore, the I^2 statistic was used to quantify the proportion of heterogeneity between the studies. In the presence of heterogeneity, a revision of included studies was carried out to assess the main reasons explaining the phenomenon and, therefore, a subgroup analysis was performed. Only if this attempt failed, a random effects model was employed, in order to minimize the impact of heterogeneity. We preferred a fixed effects model if less than 4 studies per outcome were included in the analysis[23].

The degrees of freedom (df) were reported for each analysis. We evaluated the quality of enrolled studies by the Jadad scale[24] for randomized clinical trials (RCTs) or by the Quality Assessment Tool for Case Series Studies (QATCSS) of the National Institutes of Health[25] for nonrandomized, open label pilot studies. Finally, when comparison between two groups (probiotics *vs* placebo) was performed, we drew funnel plots and applied Egger's regression method to estimate the asymmetry of the funnel plots, considering non-statistically significant results as absence of publication bias[26].

RESULTS

Study selection

The literature search found 1537 articles overall. After study selection, reported in detail in Figure 1, 11 studies were eligible for the analysis[27-37]. Only 7 of them were RCTs[27,29,30,32,33,36,37]. A total of 517 *H. pylori*-infected patients were recruited. Of these, 114 received a placebo treatment and served as a control group, and the remaining 403 had probiotic

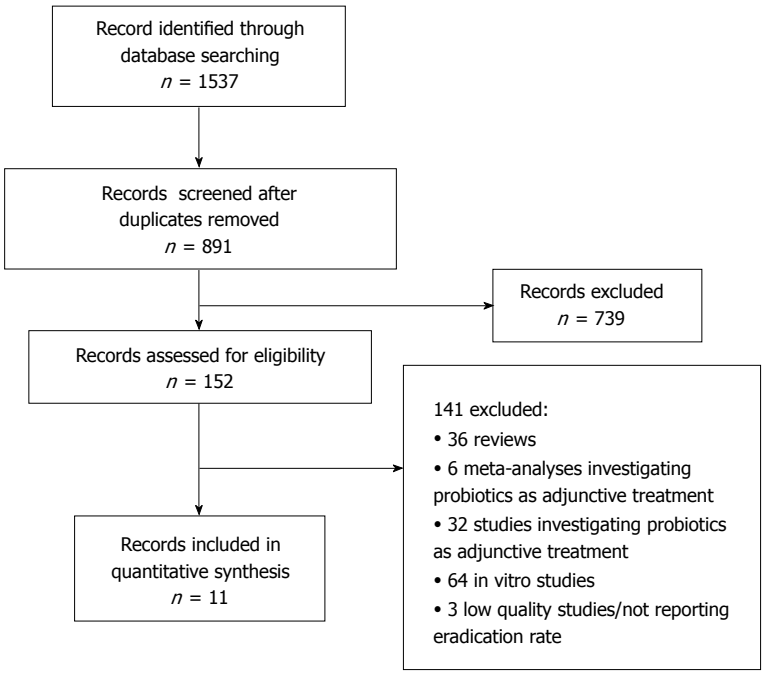

Figure 1 Flowchart showing the process of study selection for the systematic review.

supplementation. In all studies except 2, the diagnosis was achieved by UBT[27,37], but in most cases the initial diagnosis was established by the combination of more than one test, including UBT, upper endoscopy with histology or rapid urease test, serology or stool antigen test (SAT). The verification of eradication of treatment was performed by UBT in all but 2[27,37], which used SAT both for diagnosis and eradication control. Details of the cut-offs used for diagnosis and timing of UBT are reported in Table 1.

Only 3 studies were conducted on the pediatric population[30,35,37]. In most cases (7), a lactobacilli-based formulation was employed, while only 2 studies administered *Saccharomyces boulardii*[28,35] and 3 investigated probiotic multistrain formulations[31,33,36]. Of note, only in 1 study[28] was probiotics given in combination with proton pump inhibitor. The duration of probiotic supplementation varied across the studies, from 10 d to 1 year. Quality assessment is reported in Table 2.

Overall effectiveness of probiotics in eradicating H. pylori

In the 11 selected studies, probiotics eradicated *H. pylori* in 50 out of 403 cases. The mean weighted eradication rate was 14%, with a 95%CI of 2%-25% (df = 4, P = 0.02). In 6 studies, probiotic treatment was unsuccessful[29,31,32,34,35,37], while the highest percentage of eradication (32.5%) was achieved in an Italian study[33]. The forest plot of such analysis is displayed in Figure 2A.

Further, we performed a sub-analysis comparing the success rate in RCT *vs* non-randomized studies (Figure 2B). The pooled rate was 14% for RCT (95%CI: 1%-27%, df = 3, P = 0.04) and 14% for non-randomized trials (95%CI: 0%-44%, P = 0.34). No difference was found between these two groups (P = 0.99).

Eradication rate according to the probiotic strain

Most of studies investigated a probiotic formulation based on a single lactobacilli strain (further details of species are listed in Table 1)[27-30,32,34,35]. Lactobacilli eradicated the bacterium in 30 out of 235 patients, with a mean weighted rate of 16% (95%CI: 1%-31%, df = 2). Multistrain combinations[31,33,36] were effective in 14 out of 105 patients, with a pooled eradication rate of 14% (95%CI: 0%-43%, df = 1). In the two studies evaluating *Saccharomyces boulardii*[30,37], the treatment was successful in 6 out of 63 subjects (pooled rate of 12%, 95%CI: 0%-29%). We did not find any statistically significant difference among these three formulations (P = 0.94). The forest plot of this analysis is reported in Figure 3.

Probiotics vs placebo in the eradication of H. pylori

Six RCTs[27,29,32,33,36,37] compared probiotics to a placebo (see Figure 4). In total, probiotics eradicated the bacterium in 38 out of 238 patients (15.9%), while placebo alone did not achieve any success (0 out of 114, 0%). The analysis, reported in Figure 4, provided an OR of 7.91 in favor of probiotics, with a 95%CI of 2.97-21.05. In this case, we used a fixed effects model since heterogeneity was absent (χ^2 = 0.75, df = 2, P = 0.69). A funnel plot, reported in Figure 5, showed that a possible bias could be detected, as confirmed by Egger's test (P = 0.02). However, the low number of included studies and the presence of 0% eradication rates (which are void for the test) imply that the test has a low statistical power, and therefore the possibility

Table 1 Main characteristics of the studies included in the quantitative analysis

Ref.	Nation	Age and sex	Probiotic strain and dose	Diagnosis	Control of eradication	Eradication rate % (n/N)
Boonyaritichaikij *et al*[27], 2009	Japan	62 ± 14 yr Male sex: 54.5%	Cheese with *L. gasseri* OLL2716 5 × 10^8 CFU/g for 12 mo	SAT	SAT after 12 mo	Probiotic: 29.3% (24/82) Placebo: 0% (0/6)
Dore *et al*[28], 2014	Italy	Mean age: 51 yr (range, 21-68) Male sex: 13.6%	*L. reuteri* 10^8 CFU/tablet bid + Pantoprazole 20 mg bid for 60 d	UBT	UBT after 30-40 d	14.3% (3/21)
Francavilla *et al*[29], 2008	Italy	53.3 ± 13.3 yr (probiotics) 52.4 ± 13.1 yr (placebo) Male sex: 57.5%	*L. reuteri* ATCC55730 10^8 CFU/tablet bid for 28 d	UBT (cut-off 3.5%), SAT, RUT, histology	UBT after 4 wk	Probiotic: 0% (0/20) Placebo: 0% (0/20)
Gotteland *et al*[30], 2005	Chile	8.5 ± 1.7 Male sex: 49.6%	*L. acidophilus* LB 10^9/tablet bid or *S. boulardii* 250 mg + inulin 5 g bid for 8 wk	UBT (cut-off 5‰)	UBT after 1 d	9.3% (9/97) *L. acidophilus* 6.5% (3/46) *S. boulardii* 11.8% (6/51)
Myllyluoma *et al*[31], 2007	Finland	Mean age: 51 yr (range, 40-69)	Multi-strain (*L. rhamnosus* GG, *L. rhamnosus* LC705, *P. freudenreichii* JS, *B. lactis* Bb12) 2.5 × 10^9 CFU/day for 8 wk	UBT (cut-off 2.2%), RUT, histology	UBT after 8 wk	0% (0/6)
Pantoflickova *et al*[32], 2003	Switzerland	25 ± 5 yr Male sex: 50%	*L. johnsonii* bid for 3 wk, then once daily for 13 wk	UBT (cutoff 5%), histology, culture, RUT, serology	UBT, culture at the end of treatment	Probiotic: 0% (0/25) Placebo: 0% (0/25)
Rosania *et al*[33], 2012	Italy	52.4 ± 21.7 yr (probiotics) 48.7 ± 25.3 yr (placebo) Male sex: 42.5%	Multi-strain (*S. termophilus*, *L. acidophilus*, *B. longum*, *L. plantarum*, *B. brevis*, *L. paracasei*, *B. infantis*, *L. delbrueckii*) 1800 × 10^9/d for 10 d	UBT (cut-off 4%)	UBT after 4 wk	Probiotic: 32.5% (13/40) Placebo: 0% (0/40)
Sakamoto *et al*[34], 2001	Japan	50.1 ± 7.4 yr Male sex: 93.1%	Yoghurt + *L. gasseri* OLL2716 1-1.4 × 10^7 CFU/g bid for 8 wk	UBT (cut-off 5%)	UBT after 9 wk	0% (0/29)
Shimizu *et al*[35], 2002	Japan	Mean age: 12.1 yr (range, 7.4-15.8) Male sex: 41.7%	Yoghurt + *L. gasseri* OLL2716 1-1.4 × 10^7 CFU/g bid for 8 wk	SAT, UBT	SAT, UBT after 4 and 10 wk	0% (0/12)
Wang *et al*[36], 2004	China	Not available	Multi-strain yoghurt (*L. acidophilus* La5, *B. lactis* Bb12, *L. bulgaricus*, *S. termophilus*) > 10^7 bacteria/mL for 6 wk	UBT (cut-off 3.5%), histology	UBT after 8 wk	Probiotic: 1.7% (1/59) Placebo: 0% (0/11)
Namkin *et al*[37], 2016	Iran	Age range of 9-12 yr Male sex: 20.8%	*S. boulardii* 250 mg/d for 1 mo	SAT	SAT after 8 wk	Probiotic: 0% (0/12) Placebo: 0% (0/12)

CFU: Colony forming units; RUT: Rapid urease test; SAT: Stool antigen test; UBT: Urea breath test.

Table 2 Quality assessment according to the type of studies

Ref.	Type of study	Jadad score[1]	QATCSS score[2]
Boonyaritichaikij *et al*[27], 2009	Randomized, single blind placebo-controlled, pilot	3	NA
Dore *et al*[28], 2014	Prospective, single center, open label pilot study	NA	8
Francavilla *et al*[29], 2008	Randomized, double blind placebo-controlled	4	NA
Gotteland *et al*[30], 2005	Randomized, open study	3	NA
Myllyluoma *et al*[31], 2007	Prospective, single center, open label pilot study	NA	7
Pantoflickova *et al*[32], 2003	Randomized, double blind placebo-controlled	4	NA
Rosania *et al*[33], 2012	Randomized, double blind placebo-controlled	4	NA
Sakamoto *et al*[34], 2001	Single center, open label pilot study	NA	6
Shimizu *et al*[35], 2002	Single center, open label pilot study	NA	6
Wang *et al*[36], 2004	Randomized, double blind placebo-controlled	2	NA
Namkin *et al*[37], 2016	Randomized, double blind placebo-controlled	5	NA

[1]Jadad scale reaches a maximum score of 5; [2]QATCSS reaches a maximum score of 9. NA: Not applicable.

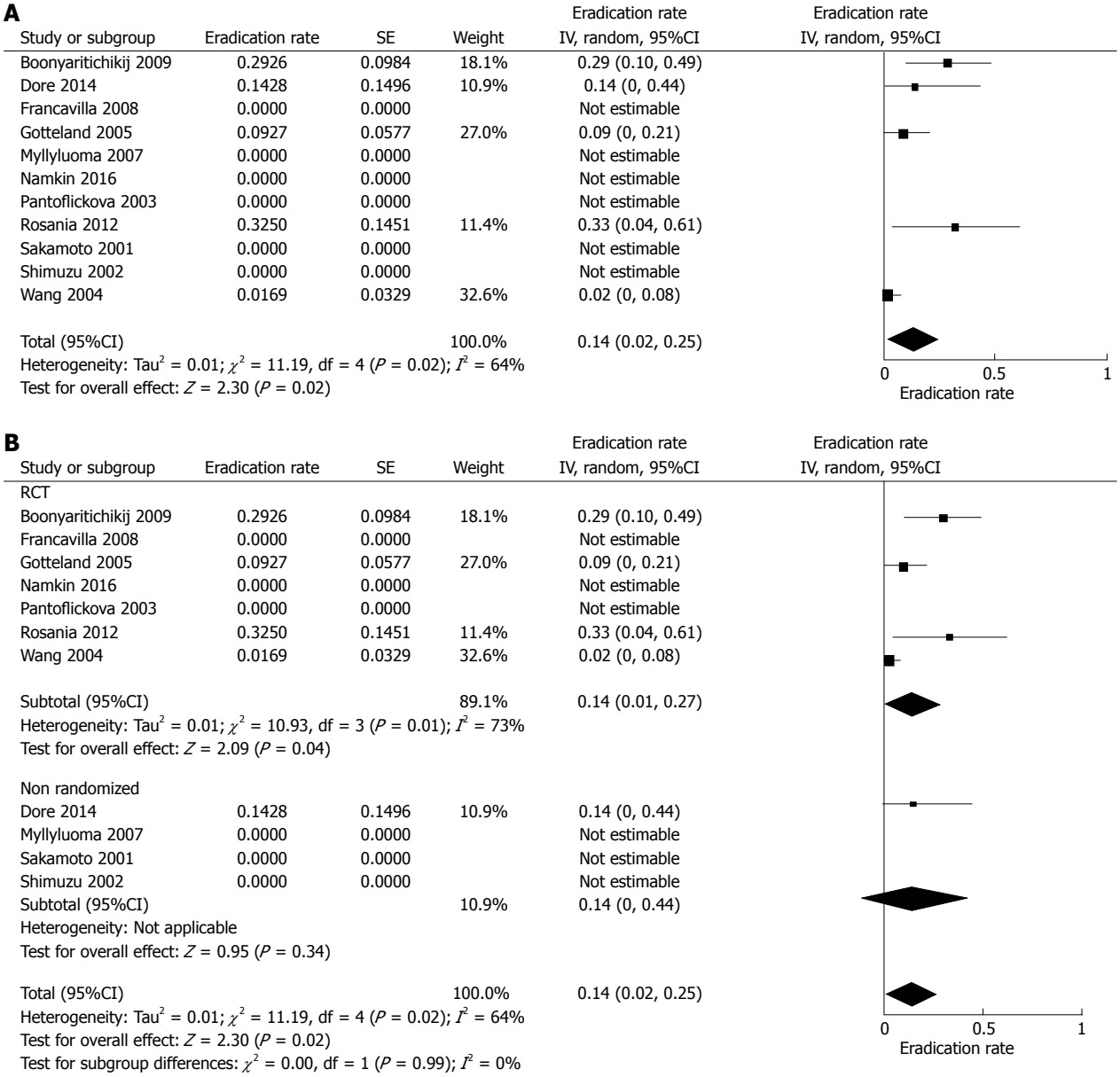

Figure 2 Mean eradication rate of probiotics for *H. pylori* infection (A). B: A sub-analysis according to the type of studies (randomized controlled trials (RCTs) *vs* open label studies) is reported.

of bias is questionable anyway.

Variations in delta values for UBT

We aimed to evaluate whether probiotics' administration alone could reduce the expired ^{14}C-marked CO_2 during the UBT. Six studies provided sufficient data (delta values expressed as ‰) to perform such analysis[29,30,33-36]. In two studies, delta values for placebo were reported[29,33].

Overall, probiotics induced a statistically significant mean reduction in delta values of 8.61‰ (95%CI: 5.88-11.34, df = 6) which was statistically significant. On the other hand, placebo implied a reduction of 0.19‰, which was not statistically significant (95%CI: -5.16-5.53, P = 0.94, df = 1). The test for subgroup differences demonstrated that probiotics significantly reduced delta compared to placebo (P = 0.006). In this analysis, despite a high heterogeneity (χ^2 = 47.08, df = 8, P < 0.001, I^2 = 83%) we used a fixed effects model since the number of included studies was low and the heterogeneity could be explained by the different type of probiotics and the different study design of enclosed trials. The forest plot of this analysis is reported in Figure 6.

Adverse events

Only 3 studies described adverse events during probiotic administration[28,30,37], and only 1 case of side effect was reported in 39 treated patients, with a pooled prevalence of 8% (95%CI: 0%-39%, P = 0.59). In only 1 study[37], side effect rate was reported both for placebo and probiotic groups. In this case, the

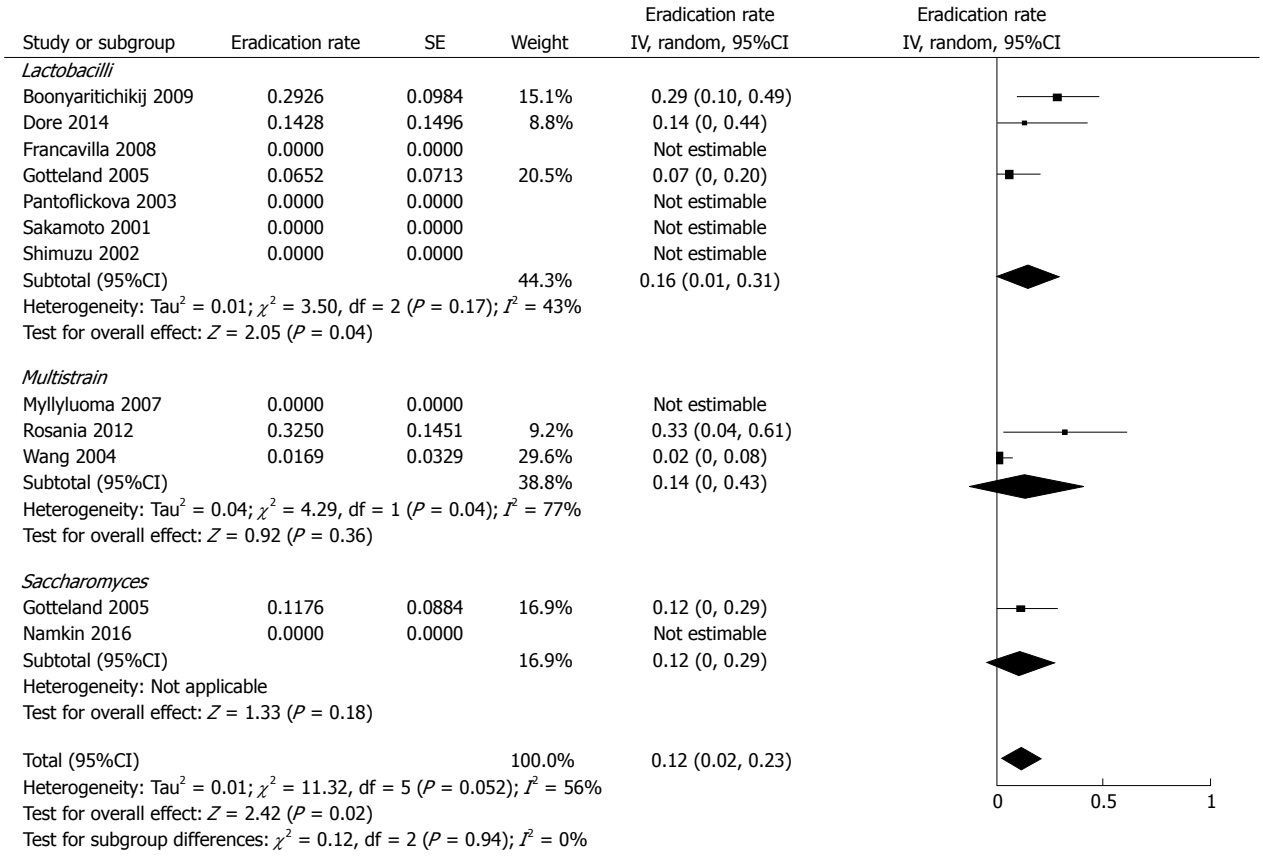

Figure 3 Sub-analysis of probiotics' effectiveness in *H. pylori* eradication according to the strain.

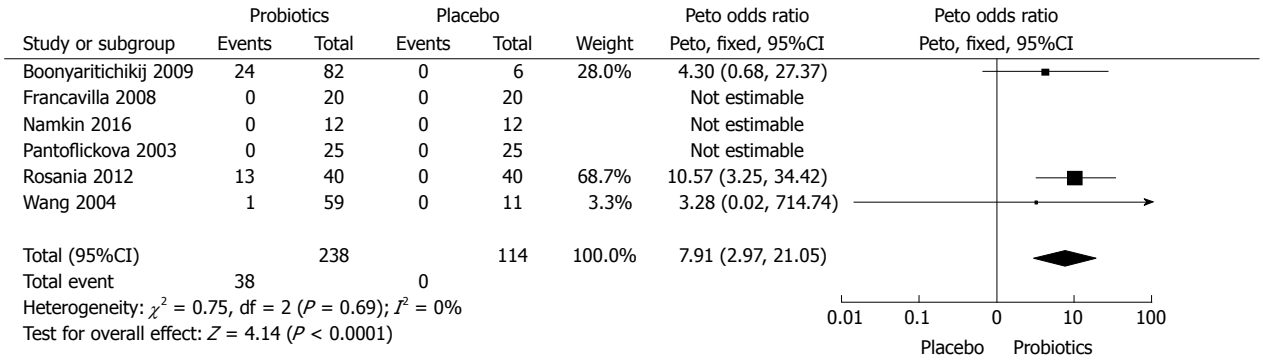

Figure 4 Meta-analysis comparing the eradication rate of probiotics against placebo.

meta-analysis did not show any difference between the two groups (OR = 1, 95%CI: 0.06-18.08, *P* = 1).

DISCUSSION

Despite several lines of evidence in the literature having demonstrated a consistent role of probiotics as adjunctive treatment for *H. pylori* eradication[38], national and international guidelines do not address a uniform consensus about their clinical application. The last Maastricht guidelines state that certain probiotics may have a beneficial impact on the eradication[39]. Similarly, Italian guidelines advise their use since they may reduce antibiotics-related side effects[40]. On the other hand, Toronto guidelines discourage routine probiotic administration in order to reduce side effects and improve the efficacy, since clinical trials and meta-analyses are characterized by low quality[41].

The most important issue that sets a limit to draw conclusions about the effects of probiotics in the treatment of *H. pylori* is that they have been considered only as an adjunctive treatment to antibiotics. In this context, probiotics demonstrated effectiveness mainly in reducing adverse events (especially diarrhea). However, these studies did not provide adequate evidence regarding a direct role in the eradication. Few studies have focused probiotic alone activity on bacteriotherapy in this field and, to date, this is the first systematic review on this topic.

In our analysis, the exclusive inclusion of studies

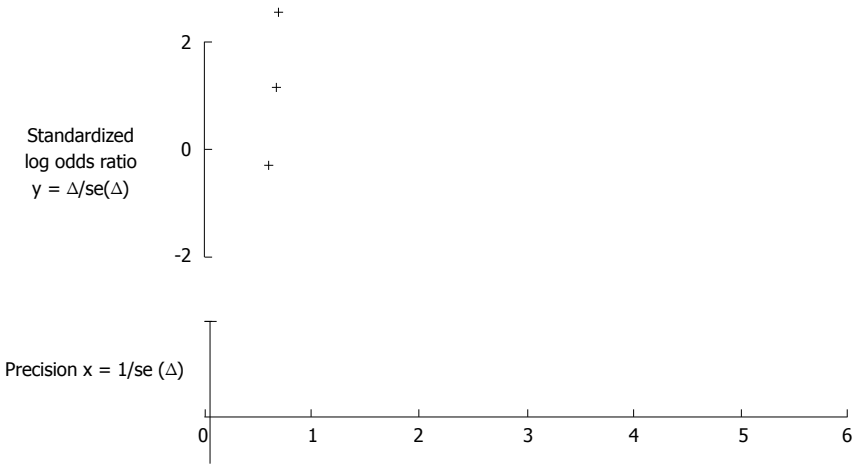

Figure 5 Funnel plot of the meta-analysis comparing the eradication rate of probiotics against placebo.

Study or subgroup	Before Mean	Before SD	Before Total	After Mean	After SD	After Total	Weight	Mean difference IV, fixed, 95%CI
Probiotics								
Francavilla 2008	33.8	15.0	20	27.3	12.1	20	8.3%	6.50 (-1.95, 14.95)
Gotteland lactobacilli	33.4	19.3	46	32.7	15.2	46	11.7%	0.70 (-6.40, 7.80)
Gotteland Saccharomyces	31.2	17.4	51	27.5	18.2	51	12.4%	3.70 (-3.21, 10.61)
Rosania 2012	39.5	19.3	40	12.5	8.7	40	13.7%	27.00 (20.44, 33.56)
Sakamoto 2001	26.6	13.7	29	20.9	11.8	29	13.7%	5.70 (-0.88, 12.28)
Shimuzu 2002	28.1	14.2	12	26.2	13.1	12	5.0%	1.90 (-9.03, 12.83)
Wang 2004	36.2	19.4	59	28.2	15.8	59	14.5%	8.00 (1.62, 14.38)
Subtotal (95%CI)			257			257	79.3%	8.61 (5.88, 11.34)
Heterogeneity: χ^2 = 39.37, df = 6 (P < 0.00001); I^2 = 85%								
Test for overall effect: Z = 6.18 (P < 0.00001)								
Placebo								
Francavilla 2008	35.8	15.5	20	37.3	16.2	20	6.1%	-1.50 (-11.33, 8.33)
Rosania 2012	33.5	15.5	40	32.6	13.5	40	14.6%	0.90 (-5.47, 7.27)
Subtotal (95%CI)			60			60	20.7%	0.19 (-5.16, 5.53)
Heterogeneity: χ^2 = 0.16, df = 1 (P = 0.69); I^2 = 0%								
Test for overall effect: Z = 0.07 (P = 0.94)								
Total (95%CI)			317			317	100.0%	6.86 (4.43, 9.30)
Heterogeneity: χ^2 = 47.08, df = 8 (P < 0.00001); I^2 = 83%								
Test for overall effect: Z = 5.53 (P < 0.00001)								
Test for subgroup differences: χ^2 = 7.55, df = 1 (P = 0.006); I^2 = 86.8%								

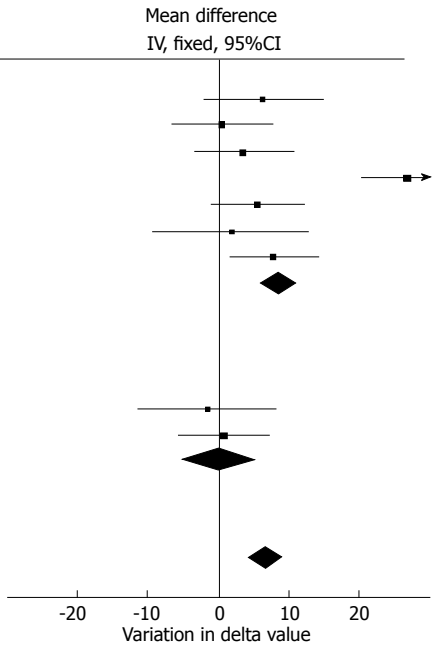

Figure 6 Variations of delta value for urea breath test before and after the treatment, both for probiotics and placebo.

using probiotics alone allowed us to draw more solid conclusions about the role of probiotics, since we removed the interference of factors and bias related to antibiotics such as inhomogeneous resistance pattern, variations in doses and administration modalities, patient compliance and adverse events. On the other hand, our analysis implied other limitations, such as the low number of enrolled patients, the differences of administered probiotic strains and the lack of randomization and/or a placebo arm as control group. For this reason, we attempted to limit these sources of heterogeneity by adding subgroup analyses and by choosing a random effect model heterogeneity that was high, a strategy that can minimize this phenomenon[23]. Finally and unfortunately, none of the included studies reported any data about smoking habits nor on alcohol assumption. Therefore, we were unable to perform a sub-analysis. This is another drawback, since it is known that such factors could influence the eradication. However, most of studies were conducted in pediatric populations, so that we may assume that such cases patients did not consume alcohol nor cigarettes.

The first relevant finding of this review is that probiotics alone may eradicate *H. pylori*, in 14%. From a clinical point of view, this is an unsatisfactory rate; however, taking into account that this percentage is considerably higher than placebo (0%, with a Peto OR = 7.91; Figure 4), we could assume that probiotic direct antibacterial action against *H. pylori* is consistent. Our analysis failed to ascertain whether some formulations may be more effective than others, but this limitation is due to the low number of included studies. Indeed, better outcome (32.5% of successful eradication) was achieved in the study which employed a multistrain

combination with the highest bacterial charge[33]. On the other hand, in 4 out of 7 studies using a single lactobacillus strain, no eradication was recorded. These observations may suggest that an association of more bacterial species could be more effective[42]. One study explored the effect of *Saccharomyces boulardii*, a yeast species, demonstrating a success rate of 11.8% and, thus, indicating a reliable performance in *H. pylori* gastritis[43,44].

The second important result concerns the variations in delta values for UBT. Indeed, as shown in Figure 6, in all studies, a reduction of delta values was observed in the probiotic arm, while delta values remained stable in subjects assuming placebo. This result is in agreement with evidence from the literature[45,46] and may suggest that probiotics could reduce the bacterial load in any case, despite a complete eradication not being obtained[47,48]. Indeed, labeled CO_2 in the expirate is considered as an indirect indicator of the density of gastric *H. pylori* colonization[49,50]. A probiotic-induced intragastric bacterial load reduction has been confirmed by histological semiquantitative analysis in some included studies[31] and even by a study, which used an original assessment of bacterial stool antigen[29,51].

In conclusion, preliminary data show that a primary therapeutic effect of probiotics may be hypothesized for *H. pylori*, but the low number of studies, their inhomogeneity in the design and the low number of enrolled patients are a critical limit to drawing evidence-based conclusions. However, the modulation of gastric microbiota could represent an intriguing aspect, since it does not imply the drawback of antibiotics (induction of dysbiosis, side effects) and is safe and probably more acceptable for patients[11,52].

ARTICLE HIGHLIGHTS

Research background

Probiotics have been largely used as adjunctive treatment for *Helicobacter pylori* (*H. pylori*) eradication, showing good results.

Research motivation

Until now, meta-analyses have investigated probiotic effects on *H. pylori* only in association with antibiotics. Therefore, our aim was to perform a systematic review with pooled data analysis regarding this uninvestigated topic.

Research objectives

The objective was to perform a meta-analysis aiming to calculate a pooled eradication rate for probiotic monotherapy, overall and according to the strain.

Research methods

Article search and selection was conducted according to the PRISMA criteria. We performed a pooled-data analysis using to the inverse variance method to calculate the mean weighted eradication rate. Peto odd ratio (OR) was calculated for the comparison "probiotics *vs* placebo". For continuous variables (delta value of urea breath test), we entered mean, standard deviations and sample size in order to calculate the weighted mean difference.

Research results

We found that probiotic monotherapy may eradicate *H. pylori* in 14% of cases. Lactobacilli, *Saccharomyces boulardii* and multistrain combinations eradicated the bacterium with a rate of 16%, 12% and 14%, respectively. Probiotics were significantly more effective than placebo (OR = 7.91). Moreover, probiotics were able to reduce delta values in the expirate of urea breath test.

Research conclusions

The eradication rate of probiotics' monotherapy is disappointing; however, our meta-analysis showed that, in some cases, they are able to defeat the bacterium. They compete with *H. pylori* for host surface receptors and, thereby, inhibit its adhesion to epithelial cells. Furthermore, it has been demonstrated that probiotics could hamper *H. pylori* urease activity. On these bases, since probiotics administration does not carry the risk of antibiotic resistance, it could represent an optimal strategy in selected cases.

Research perspectives

Further studies on large sample size are necessary to draw more solid conclusions about a direct inhibitory effect of probiotics on *H. pylori*.

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CASE REPORT

Long-term survival after gastrectomy and metastasectomy for gastric cancer with synchronous bone metastasis

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Abstract

Bone metastasis is a rare event in patients with gastric cancer, but pathologic fracture, paralysis, pain and hematological disorders associated with the bone metastasis may influence the quality of life. We report herein the case of a 53-year-old man who presented with primary remnant gastric cancer with bone metastasis. The patient requested further investigations after detection of a metastatic lesion in the 2nd lumbar vertebra during evaluation for back pain that had persisted for 3 mo. No other metastatic lesions were detected. He underwent total gastrectomy and palliative metastasectomy to aid in reduction of symptoms, and he received combination chemotherapy with tegafur (S-1) and cisplatin. The patient survived for about 60 mo after surgery. Currently, there is no treatment guideline for gastric cancer with bone metastasis, and we believe that gastrectomy plus metastasectomy may be an effective therapeutic option for improving quality

of life and survival in patients with resectable primary gastric cancer and bone metastasis.

Key words: Stomach neoplasms; Gastrectomy; Bone neoplasms; Neoplasm metastasis; Metastasectomy

Core tip: Gastrectomy and metastasectomy may be an effective therapeutic option for improving quality of life and survival in selected gastric cancer patients with bone metastasis. Favorable factors, such as resectable solitary bone lesions, good performance status and normal serum carcinoembryonic antigen levels, should be utilized to stratify and select patients who will be good candidates for surgery.

Choi YJ, Kim DH, Han HS, Han JH, Son SM, Kim DS, Yun HY. Long-term survival after gastrectomy and metastasectomy for gastric cancer with synchronous bone metastasis. *World J Gastroenterol* 2018; 24(1): 150-156 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i1/150.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i1.150

INTRODUCTION

The survival rate associated with gastric cancers has improved due to improvements in diagnostic technology, surgery, adjuvant therapy and increased detection at early stages. Although the incidence of gastric cancer has gradually declined, it remains the second most common cancer in South Korea, after thyroid cancer, according to the Korea Cancer Registry statistics for the year 2010. It is also the third leading cause of cancer-related death[1].

A substantial proportion of patients are diagnosed at an advanced stage with synchronous distant metastases. Treating such patients is a therapeutic challenge for physicians, since it is generally accepted that such patients have incurable disease and that treatment is administered with a noncurative intent. Distant metastasis in gastric cancer patients is known to be one of the most important prognostic risk factors, with associated parameters like depth of invasion and lymph node metastasis[2]. Bone metastasis is more commonly observed in other cancer types, like cancers of the breast, lung and prostate, but is rather rare in gastric cancer[3].

Gastric cancer with bone metastasis is associated with poor prognosis[4,5]. Moreover, it is related to pathologic fracture, paralysis, pain and hematological disorders, which may influence quality of life and need for further treatment. Disease management in such cases is a challenge for physicians, since no guidelines exist for treatment of patients with gastric cancer and associated bone metastasis. We present here a case of long-term survival after completion of total gastrectomy and metastasectomy for primary remnant gastric cancer (RGC) with bone metastasis. We also include a review of the relevant literature.

CASE REPORT

A 53-year-old male patient, who had complained of back pain, was found to have a mass in the 2nd lumbar vertebra by magnetic resonance imaging (MRI), and was transferred to our institution for further evaluation. He had no significant medical history, except for having undergone gastrectomy due to a gastric ulcer 15 years prior. At the onset, blood biochemical parameters were within normal range (alkaline phosphatase: 141 IU/L, normal range: 40-145 IU/L; calcium: 9.5 mg/dL, normal range: 8.2-10.8 mg/dL), as were the levels of carcinoembryonic antigen (CEA; 2.26 ng/mL, normal range: < 5.0 ng/mL), cancer antigen (CA) 19-9 (12.25 U/mL, normal range: < 37 U/mL) and lactate dehydrogenase (LDH; 152 IU/L, normal range: 70-178 IU/L). The MRI showed a suspicious metastatic lesion in the 2nd lumbar vertebra with enhancing signal intensity on a T2-weighted image, and bone scans showed a focal hot uptake at the 2nd lumbar vertebra (Figure 1A-C). A bone biopsy taken at the 2nd lumbar vertebra was diagnosed as metastatic adenocarcinoma. Gastroduodenoscopic findings showed a Borrmann type III lesion at the remnant stomach lesser curvature (Figure 1D), which was diagnosed as poorly differentiated adenocarcinoma by biopsy. Serum fasting gastrin level was within normal range, and Giemsa staining did not demonstrate *Helicobacter pylori* (*H. pylori*) infection in the gastric biopsy specimen. No other metastatic lesions were detected on chest computed tomography (CT) and abdominal pelvic CT scans.

The patient underwent total gastrectomy with Roux-en-Y esophagojejunostomy and lumbar vertebrae metastasectomy (Figure 2A and B). Pathologic findings after the surgery described poorly differentiated adenocarcinoma, with invasion of serosa and massive lymphovascular invasion. Five metastatic lymph nodes were observed among the nine regional lymph nodes examined. Microscopically, the tumor consisted of solid nests of poorly differentiated tumor cells having ovoid nuclei and indistinct cytoplasm. Immunohistochemistry showed that the tumor cells exhibited diffuse immunoreactivity for cytokeratin AE1/AE3, but were negative for synaptophysin and vimentin, a test for neurofilament immunoreactivity that has been identified in most neuroendocrine tumors. These findings support the diagnosis of poorly differentiated adenocarcinoma (Figure 3A-D).

Microscopically, the tumors of lumbar vertebrae were identified as poorly differentiated, with histologic and immunohistochemical features identical to those of the carcinoma of the stomach (Figure 3E-H). Bone marrow was also obtained from the patient's lumbar spine during surgery, and no cancer cells were

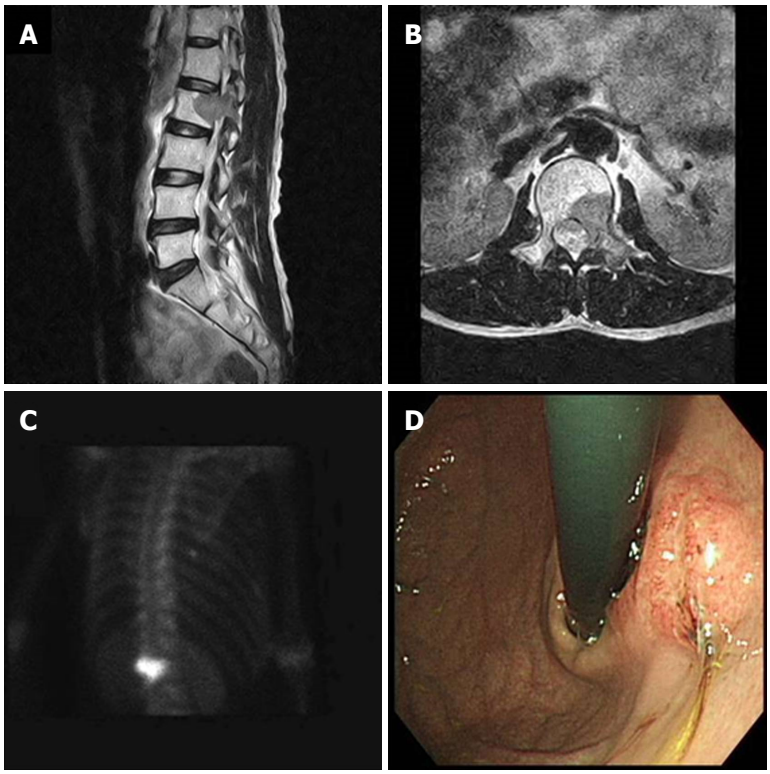

Figure 1 Radiologic findings of the 2nd lumbar vertebra and the endoscopic finding of gastric cancer. A and B: Bone magnetic resonance imaging showing bone marrow signal change and soft tissue formation at the 2nd lumbar vertebra that extended to the transverse process and the back muscle; C: Bone scan demonstrating 99m Tc-HDP 25mCi uptake in the 2nd lumbar vertebra; D: Endoscopy revealing a 3-cm ulceroinfiltrative mass on the lesser curvature of the high body of the remnant stomach.

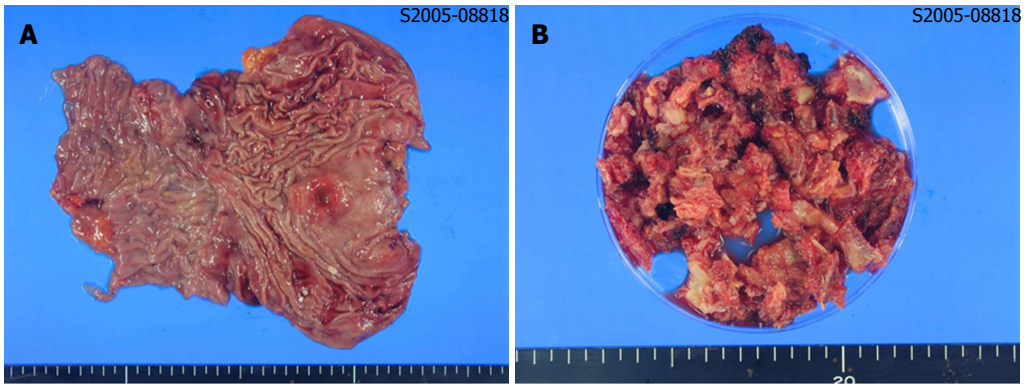

Figure 2 Macroscopic findings of resected stomach and metastatic tumor of bone. A: A 3-cm ulceroinfiltrating mass was found on the lesser curvature side of stomach. It was 4 cm distant from the proximal resection margin, and 11 cm distant from the distal resection margin; B: About 100 cc of bone and soft tissues were resected from the lumbar spine.

detected in the bone marrow aspirate. The results of postoperative pathologic findings led to a tumor-node-metastasis classification of T4aN2M1, according to the American Joint Committee on Cancer 7th edition staging manual.

The patient received palliative first-line combination chemotherapy with tegafur (S-1) and cisplatin for a year. Duration of relapse-free survival was 25 mo, when multiple metastases were found in follow-up to have developed on the right ileum and liver (Figure 4). Right ileum radiotherapy and second-line palliative combination of folinic acid, fluorouracil and oxaliplatin (known as FOLFOX chemotherapy) was administered. Third-line combination chemotherapy of folinic acid, fluorouracil and irinotecan (known as FOLFIRI) and fourth-line docetaxel chemotherapy proceeded, and the patient died 60 mo after surgery.

DISCUSSION

In this study, we report a case of RGC with synchronous isolated bone metastasis. The average interval

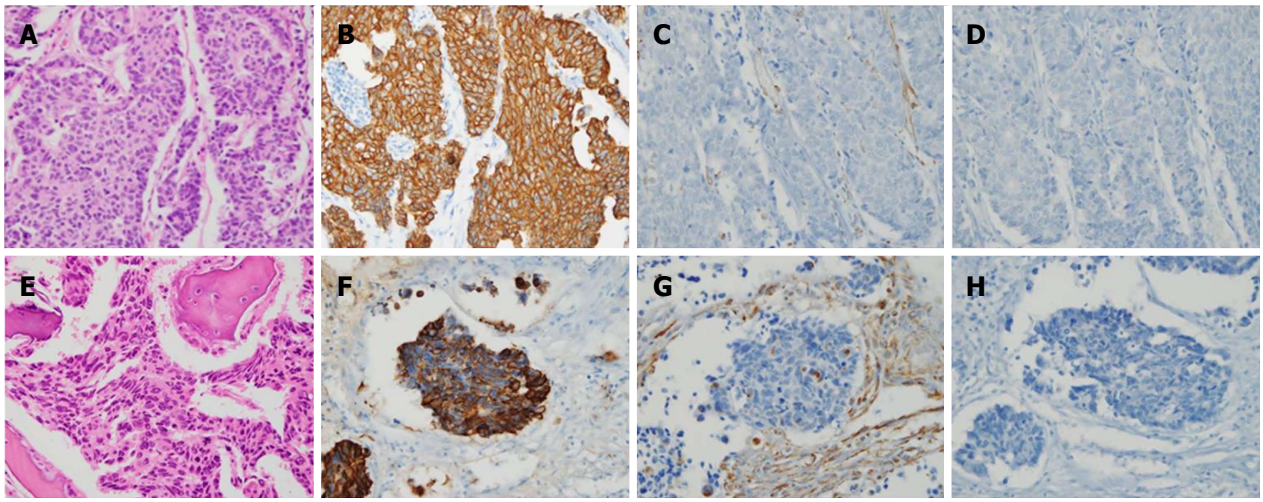

Figure 3 Histopathological and immunohistochemical findings of stomach cancer (A-D) and metastatic bone lesion (E and F). A: The stomach tumor consisted of solid nests of poorly differentiated tumor cells, with ovoid nuclei and indistinct cytoplasm (hematoxylin-eosin, × 400); B-D: Immunohistochemistry showed that the tumor cells exhibited diffuse immunoreactivity for cytokeratin AE1/AE3 (B: × 400), but were negative for vimentin (C: × 400) and synaptophysin (D: × 400), which supported the diagnosis of poorly differentiated adenocarcinoma; E: The bone tumors were identified as poorly differentiated tumors (hematoxylin-eosin, × 400), with histologic and immunohistochemical features identical to those of the carcinoma of the stomach that were positive for cytokeratin AE1/AE3 (F: × 400) and negative for vimentin (G: × 400) and synaptophysin (H: × 400).

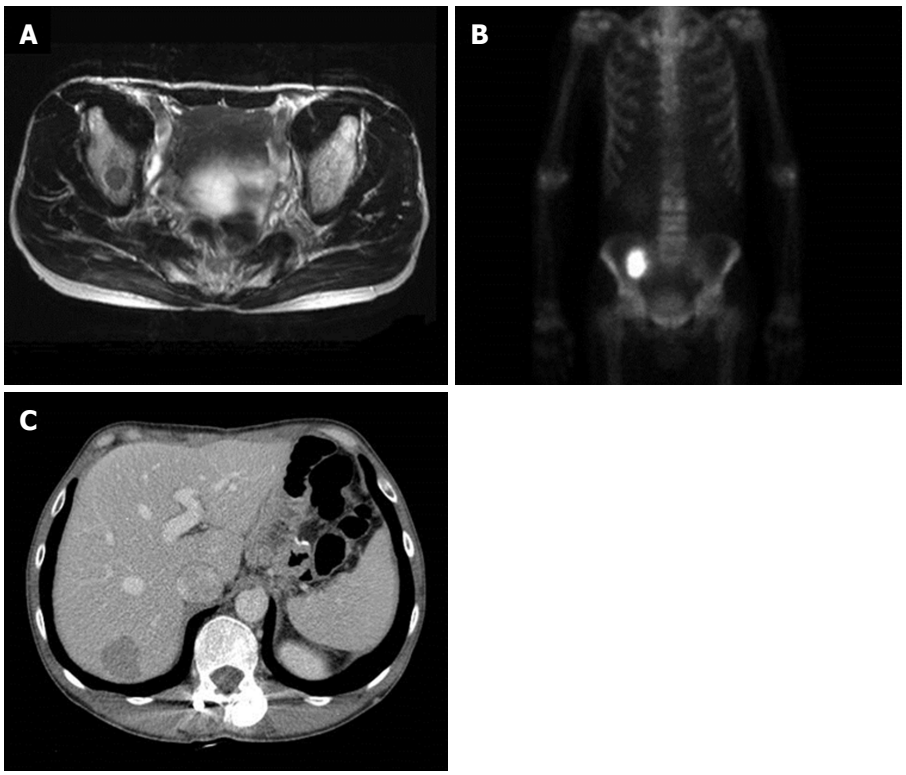

Figure 4 Findings of bone magnetic resonance imaging, bone scan and abdomen computed tomography after 25-mo of follow-up. A and B: Right ileum bone metastasis; C: Liver metastasis.

between initial distal gastrectomy and the second surgery for RGC is reported to be 22.0-34.6 years for benign disease and 6.8-18.8 years for gastric cancer[6]. As possible important factors for the pathogenesis for RGC, *H. pylori* infection, duodenogastric reflux and denervation of gastric mucosa have been considered. Also, Billroth-Ⅱ (B-Ⅱ) reconstruction is known to be more prevalent in developing RGC than Billroth-Ⅰ (B-Ⅰ) reconstruction; the possible reason is continuous bathing by duodenogastric reflux, as well as mucosal inflammation and regeneration[6,7]. In the case described herein, *H. pylori* infection was not identified. But, the previous B-Ⅱ reconstruction and persistent duodeno-gastric reflux could have been the possible cause of

RGC in this patient.

While the number of patients diagnosed with gastric cancer has increased with the development of endoscopy and mass screening tests, the survival rate of gastric cancer patients has also improved due to the advancement of diagnostic technology for early detection, surgery and postoperative adjuvant therapy. However, there is a substantial portion of the patient population with unresectable gastric cancer due to synchronous distant metastasis. It is now accepted that the presence of bone metastasis is an independent prognostic risk factor for advanced gastric cancer[8].

Gastric cancer with synchronous bone metastasis occurs rarely. Park *et al*[4] reported that synchronous bone metastases developed in about 0.9% of the total gastric cancer cases in their series. Evidence in the literature suggests that gastric cancer with synchronous bone metastasis is associated with a poor prognosis, the median survival time of which is 97 d[4]. In the majority of cases of gastric cancer with synchronous bone metastasis, the tumor is unresectable and the prognosis is very poor. There is also no established treatment guideline for these patients.

Physicians are often placed in challenging situations to manage the disease, since the difficulty is compounded by the presence of associated pathologic fractures, pain and hematologic disorders. Some studies have reported survival gain in gastric cancer patients with synchronous distant metastasis who underwent resectable gastric surgery, with or without metastasectomy[9-11]. Meta-analysis revealed that palliative gastrectomy is associated with a significant improvement in overall survival (HR = 0.62), as compared to that of patients without palliative gastrectomy[9]. Kim *et al*[11] studied the effect of gastrectomy and metastasectomy in patients with gastric cancer with distant metastases, who had previously received chemotherapy. They reported that the survival rate of patients who underwent gastrectomy plus metastasectomy was higher than that of patients who underwent debulking gastrectomy only, as well as that of patients who were administered chemotherapy only (median overall survival and 3-year survival rates reported as 28.0 mo, 15.5 mo and 9.0 mo and 42.8%, 8.1% and 3.5%, respectively). Meta-analysis also showed the survival improvement in metastatic gastric cancer patients who underwent visceral metastasectomy including liver, peritoneum and distant node. The mean increased difference in survival conferred by metastasectomy averaged between 9.3 mo and 15.7 mo[12].

However, the above studies were retrospective and selection bias cannot be ruled out. Evidence to support surgical intervention in these patients is not conclusive. There is a need for prospective randomized controlled studies to evaluate the value of resectable surgery on patients with gastric cancer with distant metastasis. Furthermore, in these studies, there was no case of metastasectomy in patients with synchronous bone metastasis. In addition, in the study conducted by Park *et al*[4] there was no evidence for curative resection of synchronous bone metastasis.

We think that for most patients with gastric cancer with synchronous bone metastases, curative resection is not an option, because the majority of bone metastases present as multiple lesions[4,13,14] and are located at unresectable anatomic locations. The most common location of bone metastasis in patients with stomach cancer is the spine, followed by the pelvis and the ribs. Hence, most patients with gastric cancer who develop bone metastases complain of back pain[15]. While there is no established treatment regimen for bone metastasis, radiation therapy is known to be effective[16,17]. In the present case, bone metastasectomy was conducted as a palliative therapy to alleviate back pain, because the solitary bone metastasis in the spine was resectable.

Clinicopathologic features associated with bone metastasis have not been fully established in the literature thus far. Sudo *et al*[18] reported that bone metastasis after surgery is observed mainly in cases of cancers involving the upper third or middle third of the stomach, which are associated with massive lymphatic invasion and poorly differentiated adenocarcinoma. In the present case, gastric cancer occurred in the remnant stomach of a patient who had undergone gastric ulcer surgery. Pathologic findings showed poorly differentiated adenocarcinoma with massive lymphovascular invasion. The mechanism by which bone metastasis develops in patients with gastric cancer is not known exactly, but York *et al*[19] observed that bone metastasis is most frequently seen in the vertebral body. Based on the location, they suggested the possibility of hematogenous spread through Batson's vertebral plexus, bypassing portal circulation.

Known factors associated with longer median survival times for the patients with bone metastasis include isolated bone metastasis, well differentiated tumors, palliative chemotherapy and zoledronic acid treatment[20]. On the other hand, high-level LDH, CEA or CA19-9, serum hypercalcemia, poor performance status, and involvement of multiple bones are associated with shorter survival times[4,5,20]. Usually, an increase of serum alkaline phosphatase levels is observed in 45.3%-66.0% of patients with bone metastasis[14,15]. In the present case, CEA, serum alkaline phosphatase and serum calcium levels were within normal range.

In the majority of cases, gastric cancer with synchronous bone metastasis is unresectable, and prognosis is usually very poor. In the present case, however, performance status was good, and the metastasis was a solitary, resectable bone lesion. These factors and aggressive palliative chemotherapy probably supported long-term survival of the patient after surgery. Therefore, we hypothesize that gastrectomy plus metastasectomy may be an effective therapeutic option for improving quality of life and survival in selected patients with bone metastasis. Favorable factors,

such as resectable solitary bone lesions, good performance status and normal serum CEA levels, should be utilized to stratify and select patients who will be good candidates for surgery.

In conclusion, aggressive local therapy, including gastrectomy with metastasectomy and palliative chemotherapy, may be an effective therapeutic option for improving survival in patients with resectable primary gastric cancer and bone metastasis.

ARTICLE HIGHLIGHTS

Case characteristics

A 53-year-old man, referred after detection of a tumorous bony lesion in the 2nd lumbar vertebra during evaluation for back pain.

Clinical diagnosis

The patient had no significant medical history, except for having undergone gastrectomy due to gastric ulcer 15 years prior.

Differential diagnosis

Primary bone neoplasm, metastatic bone tumor.

Laboratory diagnosis

Serum alkaline phosphatase, calcium, carcinoembryonic antigen (CEA), cancer antigen (CA) 19-9 and lactate dehydrogenase (LDH) levels were within normal range.

Imaging diagnosis

Magnetic resonance imaging of the spine showed a suspicious metastatic lesion in the 2nd lumbar vertebra, with enhancing signal intensity on a T2-weighted image. Gastroduodenoscopy showed a Borrmann type III lesion at the remnant stomach lesser curvature.

Pathological diagnosis

A bone biopsy taken at the 2nd lumbar vertebra led to diagnosis of metastatic adenocarcinoma. Gastric lesion was diagnosed as poorly differentiated adenocarcinoma by biopsy.

Treatment

The patient underwent total gastrectomy and lumbar vertebrae metastasectomy, followed by aggressive palliative chemotherapy.

Related reports

Gastric cancer with bone metastasis is relatively rare and associated with poor prognosis. Known factors associated with longer median survival times for patients with bone metastasis include isolated bone metastasis, well differentiated tumors, palliative chemotherapy and zoledronic acid treatment. On the other hand, high-level LDH, CEA and CA19-9, serum hypercalcemia, poor performance status, and involvement of multiple bones are associated with shorter survival times. Small studies have reported survival gain in gastric cancer patients with synchronous distant metastasis who underwent resectable gastric surgery plus metastasectomy.

Term explanation

Gastrectomy is a partial or total surgical removal of the stomach. Metastasectomy is the surgical removal of metastases, which are secondary cancerous growths that have spread from cancer originating in another organ in the body.

Experiences and lessons

Aggressive local therapy, including gastrectomy with metastasectomy and palliative chemotherapy, may be an effective therapeutic option for improving survival in patients with resectable primary gastric cancer and bone metastasis.

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CASE REPORT

Emergent single-balloon enteroscopy for overt bleeding of small intestinal vascular malformation

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Abstract

A 28-year-old man presented with anemia symptoms and intermittent tarry stool passage for three days. No stigmata of hemorrhage were identified using esophagogastroduodenoscopy, ileocolonoscopy, and contrast-enhanced computed tomography. He then developed massive tarry stool passage with profound hypovolemic shock and hypoxic respiratory failure. Emergent angiography revealed active bleeder, probably from the jejunal branches of the superior mesenteric artery, but embolization was not performed due to possible subsequent extensive bowel ischemia. His airway was secured *via* endotracheal intubation with ventilator support, and emergent antegrade single-balloon enteroscopy was performed at 8 h after clinical overt bleeding occurrence; the procedure revealed a 2-cm pulsating subepithelial tumor with a protruding

blood plug at the distal jejunum. Laparoscopic segmental resection of the jejunum with end-to-end anastomosis was performed after emergent endoscopic tattooing localization. Pathological examination revealed a vascular malformation in the submucosa with an organizing thrombus. He was uneventfully discharged 5 d later. This case report highlights the benefit of early deep enteroscopy for the treatment of small intestinal bleeding.

Key words: Early endoscopy; Small intestine; Deep enteroscopy; Device-assisted enteroscopy; Obscure gastrointestinal bleeding; Vascular malformation

Core tip: We believe that emergent deep enteroscopy performed with a secure airway and ventilator support can efficiently identify the stigmata of hemorrhage in clinical overt small intestinal bleeding and guide the operative approach.

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INTRODUCTION

The small bowel (SB) has been considered as the "dark continent" of gastrointestinal (GI) tract, and the management of SB disease is always surgical because of its inaccessibility *via* regular endoscopy. The detection and management of SB bleeding remained challenging to gastroenterologists until the introduction of device-assisted enteroscopy (DAE)[1-3]. DAE has been proposed as an efficient diagnostic tool (diagnostic yield rate from 40.7%-87.3%) and safe therapeutic (yield rate up to 48%) approach for the treatment of bleeding from SB vascular lesions[1,2,4-7]. However, it remains unknown whether the benefit provided by early endoscopic intervention in SB bleeding is similar to that provided by emergent esophagogastroduodenoscopy and colonoscopy in upper and lower GI bleeding[8]. We herein report a case of profound SB bleeding from a vascular malformation that was treated by laparoscopic surgery after localization using preoperative emergent deep enteroscopy.

CASE REPORT

A 28-year-old man without any underlying systemic disease presented to our emergency department with intermittent tarry stool passage and anemia

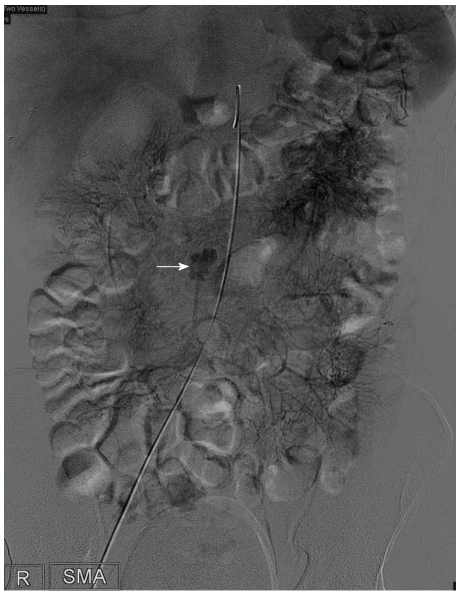

Figure 1 An active bleeder, probably from the jejunal branches, with contrast extravasation into the bowel lumen was detected by emergent angiography.

symptoms for 3 d. Physical examination showed pale conjunctiva and tachycardia (heart rate, 104 beats per min). Laboratory studies disclosed severe anemia with a hemoglobin level of 5.2 g/dL (normal range: 13-17 g/dL). Esophagogastroduodenoscopy, ileocolonoscopy, and contrast-enhanced computed tomography detected no evidence of the stigmata of active hemorrhage but identified many blood clots in the colon. He subsequently developed massive tarry stool passage, shock, and hypoxic respiratory failure despite aggressive blood transfusion resuscitation. Emergent angiography revealed an active bleeder, probably from the jejunal branches of superior mesenteric artery, with contrast extravasation into bowel lumen (Figure 1, arrow); however, embolization was not performed due to the possibility of extensive bowel ischemia. He was intubated with ventilator support and received emergent antegrade single-balloon enteroscopy (SIF-Q260; Olympus Medical systems, Tokyo, Japan) immediately (at 8 h) after resuscitation, endotracheal intubation, and angiographic localization for overt small intestinal bleeding. The procedure revealed a 2-cm pulsating subepithelial tumor with a protruding blood plug at the distal jejunum (Figure 2). Endoscopic tattooing using India ink was performed at 1 cm proximal to the lesion, and laparoscopic segmental resection of jejunum (6 cm in length) with end-to-end anastomosis was performed after emergent endoscopic localization. Pathological examination revealed a vascular malformation in the submucosa with an organizing thrombus (Figure 3, HE; original Magnification, 40 ×). His unstable hemodynamics markedly improved, and he was discharged 5 d later uneventfully.

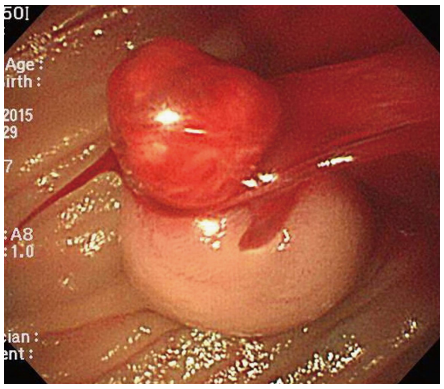

Figure 2 A 2-cm pulsating subepithelial tumor with a protruding blood plug at the distal jejunum was detected by emergent enteroscopy.

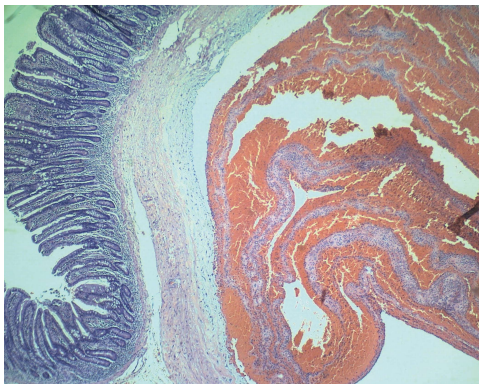

Figure 3 Vascular malformation in the submucosa with organizing thrombus in the jejunum.

DISCUSSION

Emergent deep enteroscopy is useful for the early diagnosis of small-bowel bleeding and preoperative localization of overt bleeding from small intestinal vascular malformations.

Great advents have been made in the field of GI endoscopy over the past several decades, and endoscopists have mastered the art of flexible video endoscopy for the diagnosis and treatment of upper and lower GI tract disorders. However, an endoscopic approach to the SB remains challenging because of its length and deep indwelling position in the intraperitoneal cavity. In the 20th century, thorough evaluation of the SB became possible with the development of video capsule endoscopy (VCE) and DAE[1,3]. Although VCE has been recommended as the first-line investigation for SB bleeding, DAE still has an advantage over VCE for the histopathological confirmation of SB lesions and endoscopic hemostasis[2,3]. With regard to the source of SB bleeding, vascular lesions are the most common stigmata of hemorrhage, and DAE endotherapy has been considered as an efficient and safe treatment strategy[9-12]. Additionally, the rebleeding rate of SB vascular lesions is not low, especially in the elderly among individuals with active bleeding (identified by endoscopy), a history of aortic stenosis, or those in whom the bleeding source was angioectasia; the management of rebleeding using repeated endotherapy has favorable outcomes[9,10]. Among SB vascular lesions, arteriovenous malformation bleeding can be life-threatening; hence, prompt identification and timely intervention are very important[13-16]. Although non-invasive radiological imaging or VCE is useful for the diagnosis of SB vascular lesions, lesions of smaller size or with intermittent bleeding can still be missed; meanwhile, early endoscopic intervention can be less problematic[14-16]. Nevertheless, there is scarce data regarding the timing of emergent DAE for SB bleeding. A retrospective cohort study of single-balloon enteroscopy for the management of SB bleeding did not observe a greater diagnostic or therapeutic yield in the emergent (within 24 h) enteroscopy group, but early intervention may allow for earlier stabilization and shorter hospital stays[8]. In this case report of overt bleeding from a vascular malformation in the jejunum, timely identification of the SB bleeder and preoperative localization was achieved using emergent single-balloon enteroscopy. Meanwhile, the approaching route by deep enteroscopy can be decided by pre-endoscopic angiography which assists in identification of bleeders with short endoscopy procedure time. The patient was stabilized rapidly and discharged early after laparoscopic segmental resection of the jejunal vascular malformation.

In conclusion, we successfully treated a patient with unstable hemodynamics due to overt bleeding from an SB vascular malformation using emergent preoperative deep enteroscopy. We conclude that emergent DAE is more useful than other non-invasive diagnostic modalities in the treatment of clinically overt SB bleeding; however, additional prospective studies evaluating this finding are warranted.

ARTICLE HIGHLIGHTS

Case characteristics

A young man presenting massive tarry stool passage with unstable hemodynamics.

Clinical diagnosis

Overt small intestinal bleeding.

Differential diagnosis

Vascular lesions of small intestine, Meckel's diverticulum.

Laboratory diagnosis

Severe anemia.

Imaging diagnosis

An active bleeder, probably from the jejunal branches, with contrast extravasation into the bowel lumen was detected by emergent angiography.

Pathological diagnosis

Vascular malformation in the submucosa with organizing thrombus in the

jejunum.

Treatment

Laparoscopic small intestinal resection after localization of bleeder by emergent single-balloon enteroscopy.

Related reports

Early endoscopic intervention may allow for earlier stabilization and shorter hospital stays.

Experiences and lessons

Emergent DAE is more useful than other non-invasive diagnostic modalities in the treatment of clinically overt small intestinal bleeding.

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