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EDITORIAL

Noninvasive molecular analysis of *Helicobacter pylori*: Is it time for tailored first-line therapy?

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Abstract

The main problem of *Helicobacter pylori* (*H. pylori*) infection management is linked to antibiotic resistances. This phenomenon has grown in the last decade, inducing a dramatic decline in conventional regimen effectiveness. The causes of resistance are point mutations in bacterial DNA, which interfere with antibiotic mechanism of action, especially clarithromycin and levofloxacin. Therefore, international guidelines have recently discouraged their use in areas with a relevant resistance percentage, suggesting first-line schedules with expected high eradication rates, *i.e.*, bismuth containing or non-bismuth quadruple therapies. These regimens require the daily assumption of a large number of tablets. Consequently, a complete adherence is expected only in subjects who may be motivated by the presence of major disorders. However, an incomplete adherence to antibiotic therapies may lead to resistance onset, since sub-inhibitory concentrations could stimulate the selection of resistant mutants. Of note, a recent meta-analysis suggests that susceptibility tests may be more useful for the choice of first than second-line or rescue treatment. Additionally, susceptibility guided therapy has been demonstrated to be highly effective and superior to empiric treatments by both meta-analyses and recent clinical studies. Conventional susceptibility test is represented by culture and antibiogram. However, the method is not available everywhere mainly for methodology-related factors and fails to detect hetero-resistances. Polymerase chain reaction (PCR)-based, culture-free techniques on gastric biopsy samples are accurate in finding even minimal traces of genotypic resistant strains and hetero-resistant status by the identification of specific point mutations. The need for an invasive endoscopic procedure has been the most important limit to their spread. A further step has, moreover, been the detection of point

mutations in bacterial DNA fecal samples. Few studies on clarithromycin susceptibility have shown an overall high sensitivity and specificity when compared with culture or PCR on gastric biopsies. On these bases, two commercial tests are now available although they have shown some controversial findings. A novel PCR method showed a full concordance between tissue and stool results in a preliminary experience. In conclusion, despite poor validation, there is increasing evidence of a potential availability of noninvasive investigations able to detect *H. pylori* resistances to antibiotics. These kinds of analysis are currently at a very early phase of development and caution should be paid about their clinical application. Only further studies aimed to evaluate their sensitivity and specificity will afford novel data for solid considerations. Nevertheless, noninvasive molecular tests may improve patient compliance, time/cost of infection management and therapeutic outcome. Moreover, the potential risk of a future increase of resistance to quadruple regimens as a consequence of their use on large scale and incomplete patient adherence could be avoided.

Key words: *Helicobacter pylori*; Antibiotic resistance; Noninvasive molecular test; Tailored therapy; Stool

Core tip: The main problem of *Helicobacter pylori* (*H. pylori*) infection management is linked to antibiotic resistances. They are due to point mutations in bacterial DNA. Polymerase chain reaction-based, culture-free techniques on gastric biopsy samples are accurate in finding minimal traces of genotypic resistant and hetero-resistant strains. The need for endoscopic procedure is the most important limit to their spread. Therefore, the further step has been the detection of point mutations in bacterial DNA fecal samples. There is increasing evidence of potential availability of noninvasive investigations able to detect *H. pylori* resistances to antibiotics, which may lead to tailored first-line therapies.

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INTRODUCTION

Recently, studies about *Helicobacter pylori* (*H. pylori*) infection worldwide have shown some challenging truths: (1) bacterium spreading is still ongoing[1]; (2) for no other infection such a large number of therapeutic regimens has been proposed[2]; (3) the results are controversial: the same pattern can give exciting or disappointing results depending on geographical areas[3]; and (4) although many experts claim that there are no intractable but only inadequately treated *H. pylori* strains, currently no study in the world has displayed a 100% therapeutic success rate, *i.e.,* the ideal therapy does not still exist[4].

It is undeniable that above listed problems are related to the matter of antibiotic resistances[5]. The magnitude of the phenomenon has grown in the last decade, thus both inducing a dramatic decline in the effectiveness of conventional treatment regimens and stimulating the exploration of the basis of antibiotic failure. Resistances may be divided into: (1) primary: present in subjects never treated for *H. pylori*; (2) secondary: acquired after one or more treatment schedules; and (3) hetero-resistance: coexistence of both susceptible and resistant strains in the same subject[6].

The causes of resistance are point mutations in bacterial DNA, which interfere with the mechanism of action of the different antibiotics. Clarithromycin, used for a long period as the key-antibiotic in many regimens, inhibits protein synthesis of the bacterium by acting at 23S ribosome subunit[7]. A dozen of point mutations in this site have been described worldwide. In Italy, the presence of six of them have been observed[8]. However, only three are responsible of most resistances in developed countries[7]. Interestingly, the main 23S DNA changes inducing clarithromycin resistance are dissimilar in Western and Eastern countries. These concerns depict a heterogeneous scenario of clarithromycin resistance in the different geographic areas[3]. On the other hand, levofloxacin mechanism of action involves the inhibition of gyrase enzyme with a consequent failure of bacterial DNA synthesis[6]. GyrA gene codifies for gyrase enzyme. Five point mutations in this site, able to induce resistance to this antibiotic, have been observed[9].

THERAPEUTIC STRATEGIES

On these bases, key international guideline agencies have recently discouraged the use of clarithromycin and levofloxacin in areas with a resistance rate higher than 15% in first and second-line regimens, respectively[10,11]. Nevertheless, guidelines are designed for the clinical practice within an empirical setting, *i.e.,* where susceptibility testing is unavailable, epidemiological data are taken into account to allow for the rational use of antibiotics. Therefore, suggested first line schedules are those with an expected high overall eradication rate, *i.e.,* bismuth containing quadruple therapy or non-bismuth concomitant quadruple therapy[10,11] according to Maastricht V and Toronto guidelines. However, the 2009 Asia-Pacific

guidelines suggest 14-d triple therapy or bismuth containing quadruple in first line, stating, moreover, that sequential therapy is not supported by convincing data in Asian countries[12]. On the other hand, the comparison of an old and outdated treatment *vs* a more recent and successful one should not put forward any doubt about the outcome.

A first observation which can be moved to this strategy is that these regimens require the daily assumption of a high number of tablets (14 and 8, respectively)[13]. An evident problem arising from this issue is related to the compliance of patients. A complete adherence is expected by subjects who may be motivated by the presence of major conditions, such as MALT lymphoma, family history of gastric cancer or peptic ulcer, particularly when complicated with episodes of bleeding, and the need for a concomitant long-term assumption of nonsteroidal anti-inflammatory drugs[13]. On the other hand, *H. pylori* positive dyspeptic or asymptomatic population probably may not be driven by similar motivations, given that in most cases the eradication of the bacterium is not accompanied by a tangible clinical benefit[14]. However, an incomplete adherence to an antibiotic therapy may be an important reason of resistance onset, since sub-inhibitory concentrations in the vicinity of *H. pylori* could stimulate the selection of resistant mutants[15].

Bismuth containing quadruple therapy encloses the use of tetracycline, which, currently, shows very low resistance percentages in Europe with a trend to increase from West towards East (1%-5%)[3]. However, in Asia even tetracycline resistance rates of 19% are reported[16]. Therefore, an interesting question for the future could be represented by the risk of an increase of resistance to this antibiotic due to its use on a large scale and the incomplete patient adherence to its intake[17]. On the other hand, metronidazole resistance reaches high values worldwide especially in Asian regions, where it is generally estimated not lower than 50%[3]. No data regarding bismuth resistance has been described at the best of our knowledge.

Concomitant therapy implies the use of three conventional antibiotics with the evident aim to overcome the resistance to each single drug by the overall combined effect of the regimen. Presumably, for this reason, concomitant schedule is suggested as the treatment of choice, when compared to sequential therapy[18].

A final point concerns the duration of suggested first line therapies. Commercial kit of bismuth containing quadruple therapy provides a pill number necessary for a 10-d schedule and the same period is the minimal time requested for concomitant regimen. Nevertheless, a prolongation to 14-d of both treatments seems to improve their effectiveness[10,11].

TAILORED THERAPY

A meta-analysis by Wenzhen *et al*[19] carried out in 2010, suggested that first-line susceptibility-guided triple therapy achieved a significantly higher *H. pylori* eradication than standard triple therapy. A successive similar analysis by López-Góngora *et al*[20] was performed in 2015 and included twelve studies, clearly demonstrating that susceptibility-guided therapy was superior to empirical one. However, the same study also revealed no significant differences between second-line susceptibility-guided and empirical therapies. Moreover, the low number of studies and their high heterogeneity did not allow drawing any conclusion for rescue treatments, despite current guidelines recommend culture and antibiotic susceptibility test after first and second-line regimen failure. An interesting and original learning could emerge from the data of this last meta-analysis, *i.e.,* susceptibility tests may be more likely useful for the choice of first than second-line or rescue treatment. Two recent studies performed in 2016 in Poland and China confirmed the excellent performance of tailored therapy as first line approach. Indeed, a Polish trial demonstrated that a culture guided triple therapy in first line may achieve the 95.5% and 96.6% of success rate, at per protocol and intention to treat analysis, respectively[21]. Additionally, Zhou *et al*[22] demonstrated that a susceptibility based treatment in first line achieved a gain of about 10% in eradication rate over empiric concomitant or triple plus bismuth regimens.

INVASIVE SUSCEPTIBILITY TESTS

Conventional susceptibility test is represented by culture and antibiogram (E-test) in *H. pylori* isolates. Nevertheless, this investigation is recommended by current guidelines only after repeated treatment failures. The reasons hampering its widespread use are mainly due to relatively high rate of false negatives, often showing a low sensitivity[23]. Moreover, the method is not available everywhere mainly for methodology-related factors (number of gastric biopsies/time-consuming endoscopic procedures, conditions/interval of biopsy sample transport, laboratory characteristics and time needed for the result of the investigation). Of note, a failure of E-test in hetero-resistance detection has been observed[24].

On these bases, in the last years, different polymerase chain reaction (PCR)-based approaches have been developed as alternative tools to bacterium culture. These techniques allow assessing, on gastric biopsy samples, point mutations responsible for antibiotic resistance[25]. PCR-based, culture-free techniques are accurate in finding even minimal traces of genotypic resistant strains as well as in detecting hetero-resistant status[26]. In detail, in our experience a post-hoc subgroup study, enrolling 146 *H. pylori* positive patients and comparing real time (RT)-PCR (genotypic) and E-test on bacterial culture (phenotypic) for clarithromycin resistance analysis, showed an

Table 1 Antibiotic susceptibility (clarithromycin): studies on *Helicobacter pylori* stool DNA by real time polymerase chain reaction

Ref.	Reference standard	Sensitivity	Specificity	Clarithromycin resistance
Scaletsky *et al*[32]	PCR on gastric biopsy	83.3%	100.0%	26.7%
[1]Vécsei *et al*[38]	Culture	89.2%	100.0%	45.1%
Noguchi *et al*[33]	Culture	NA	NA	20.4%
Rimbara *et al*[34]	Culture	96.6%	91.3%	13.3%
[1]Booka *et al*[35]	Culture	NA	NA	31.0%
Schabereiter-Gurtner *et al*[36]	Culture	98.0%	98.0%	24.4%
Fontana *et al*[37]	Culture	100.0%	100.0%	1.6%

[1]Pediatric population. NA: Not assessed.

overall prevalence of clarithromycin phenotypic resistance significantly lower than genotypic one. A concordance of 71.2% between the two methods was found. This value of concordance may be due to three main factors: the relative low sensitivity of phenotypic investigation, its lack of hetero-resistance detection and the possibility that E-test may identify resistant strains with point mutations different from that tested by RT-PCR in the study.

Molecular tests have been reported as promising approaches for resistance detection[26,27] even if they are not used in the clinical practice. The need for an invasive endoscopic procedure has been the most important limit to their spread. Therefore, many attempts have been performed in order to overcome this drawback.

NONINVASIVE MOLECULAR TESTS

Since 1996, our group demonstrated the possibility of *H. pylori* DNA isolation from stool samples[28]. Successively, other evidences confirmed this diagnostic chance[29-31]. A further step was undoubtedly represented by the detection of point mutations conferring antibiotic resistance in bacterial DNA fecal samples. Table 1 reports the main studies on antibiotic susceptibility (clarithromycin) performed on *H. pylori* stool DNA by RT-PCR[32-38]. For each study, diagnostic accuracy parameter and reference standard are reported. An overall high sensitivity and specificity has been observed when fecal molecular tests have been compared with culture and RT-PCR on gastric biopsies. This kind of investigation is of particular interest in the pediatric population for the need of limiting the use of invasive procedures. In detail, two studies[35,38] have been performed in children. Booka *et al*[35] found a 91% of diagnostic accuracy in reference to *H. pylori* antigen stool and a 31% prevalence of clarithromycin resistance in Japanese children. A similar result was obtained by Vécsei *et al*[38] in Austria with a diagnostic accuracy of 90.2% for bacterium detection compared to rapid urease test and histology as well as of 94% for clarithromycin resistance compared to culture.

On these bases two commercial tests are now available. A first noninvasive investigation using stool polymerase chain reaction (*H pylori* ClariRes assay, Ingenetix, Vienna, Austria) analyzed the A2142G mutation for clarithromycin resistance[38]. However, the presence of the other two major mutations responsible for the resistance to this antibiotic in developed countries (A2143G and A2142C) was not shown directly, but only hypothesized on the basis of some RT-PCR cycle temperature parameters. Successively, another molecular commercial test was developed, *i.e.*, the Genotype HelicoDR assay (Hain Lifescience GmbH, Nehren, Germany), which allows for the detection of molecular *H. pylori* resistances to clarithromycin and fluoroquinolones[39,40]. It identifies both the most common point mutations (A2146C, A2146G and A2147G) for clarithromycin resistance and *gyrA* gene mutations located at positions 87 (N87K) and 91 (D91N, D91G, D91Y) for fluoroquinolone one. This investigation has been used in tissue samples and only recently applied to stool *H. pylori* DNA[41]. However, a low concordance between stool and biopsy samples for clarithromycin and fluoroquinolone resistance detection was found.

At the same time of HelicoDR assay use on stool samples, we preliminarily experienced a novel RT-PCR method (THD Fecal Test, Italy) in order to investigate clarithromycin resistance mutations in bacterial stool DNA[42]. The procedure showed a full concordance between tissue and stool results in 52 consecutive patients at the first diagnosis of infection. We found A2143G mutation in 10 (19.2%), A2142G in 4 (7.7%) and A2142C in 5 (9.6%) patients with an overall clarithromycin resistance rate of 23%. Of interest, a preliminary experience "*in vitro*" demonstrated that the presence of fecal material (300 mg) increased the amount of colony forming units (CFU)/mL required to obtain a positive result of the detection of bacterial DNA. In detail, a clear positivity was reached by a concentration of 1.5×10 CFU/mL of pure bacteria and 1.5×10^3 of stool-mixed organisms. At the moment, a double-blinded study involving an adequate patient sample size is ongoing with the purpose to establish sensitivity, specificity, positive and negative predictive values of this noninvasive technique.

CONCLUSION

Despite the lack of a validation, there is increasing evidence of a potential future availability of noninvasive investigations able to detect *H. pylori* resistances to antibiotics, such as clarithromycin and quinolones, which have been commonly used until now. These techniques, when performed before a first-line therapy, could allow ascertaining a subgroup of strains still sensitive to these drugs and, therefore, patients benefiting from old regimens, whose administration

has been at the moment discouraged by current guidelines. However, non invasive molecular test are currently at a very early phase of development; therefore cautions should be paid when discussing about their possible clinical applications. Only further studies aimed to evaluate sensitivity and specificity of molecular tests will afford novel data to make more solid considerations. Indeed, a noninvasive susceptibility test may achieve some potential advantages, thus improving patient compliance, time/cost of infection management and therapeutic outcome. Finally, the potential risk of a future increase of resistance to quadruple regimens, suggested in first-line by guidelines, as a consequence of their use on a large scale and incomplete patient adherence could be avoided[16].

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REVIEW

Pathogenesis and clinical spectrum of primary sclerosing cholangitis

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Abstract

Primary sclerosing cholangitis (PSC) is a disease of the biliary tract, which has been documented in the literature since 1867. This disease has a strong predilection for affecting men and can be seen in individuals as young as 2 years of age. PSC has a strong associated with inflammatory bowel disease, more commonly with ulcerative colitis, and is also part of the clinical spectrum of IgG4-related diseases. Small-duct PSC, a variant of PSC, also has an association with inflammatory bowel disease. The exact pathogenesis of PSC is not well understood at present, however, is likely a combination of a genetic predisposition with alteration of the molecular structure of the gut. Abnormal serum liver chemistry and presence of certain autoimmune markers are usually the first indicators leading to a diagnosis of PCS, however, these may often be normal in early stages of this disease. The diagnosis is made by cholangiography, which is now considered the gold standard. PSC is a known pre-malignant condition. Such patients have an increased risk of developing cholangiocarcinoma, gallbladder neoplasia, and colon cancer. Many new treatment modalities have emerged in the recent past, including anti-tumor necrosis factor-α and anti-integrins; however, liver transplantation is the only known cure for PSC. Despite past and present research, PSC remains an enigmatic biliary disease with few viable treatment options.

Key words: Primary sclerosing cholangitis; Cholestasis; Inflammatory bowel disease; Autoimmune; Gallbladder neoplasia; Cholangiocarcinoma; IgG4 related disease; Colon cancer; Liver transplant

Core tip: Primary sclerosing cholangitis (PSC) is a fascinating disease with numerous and overlapping theorized pathogenetic models. An autoimmune etiology is in part due to its association with inflammatory bowel disease and autoimmune hepatitis, and inclusion within the IgG4 spectrum of diseases. Though PSC has been

documented in the literature for more than a century, only sparse details exist regarding its true pathogenetics and even less about successful medical therapy. More rigorous research is needed to truly understand and treat this disease entity.

Gidwaney NG, Pawa S, Das KM. Pathogenesis and clinical spectrum of primary sclerosing cholangitis. *World J Gastroenterol* 2017; 23(14): 2459-2469 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i14/2459.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i14.2459

INTRODUCTION

Primary sclerosing cholangitis (PSC) is a cholestatic liver and biliary tract disease associated with chronic inflammation of the biliary epithelium that cannot be attributed to another cause. This inflammatory process results in multifocal intra- and/or extrahepatic biliary strictures and fibrosis eventually leading to biliary cirrhosis and malignancy[1,2]. PSC often goes undiagnosed since approximately 40%-50% of patients with this disease are asymptomatic[3-5]. Fatigue, fever, jaundice, pruritus, and vague upper abdominal discomfort are the most commonly described symptoms at the time of diagnosis[3,6].

PSC has a strong association with inflammatory bowel disease (IBD)[7-9] with approximately 60%-80% of patients with PSC having coexisting ulcerative colitis (UC)[10,11].

PSC is a challenging condition whose pathogenesis continues to remain elusive despite extensive research of this disease in the 21st century. The only know cure for PSC is liver transplantation (LT) and symptomatic management with ursodeoxycholic acid and immunosuppressive agents.

Here we present a comprehensive review of the pathogenesis and clinical spectrum of PSC.

EPIDEMIOLOGY

PSC was first described in 1867 by Hoffman[12]. The incidence of PSC greatly varies from country to country but is increasing over time as evidenced by population-based studies from the United States, United Kingdom, and Northern Europe[13]. This variability may be related to human leukocyte antigen (HLA)-susceptibility among different ethnic groups and also varying frequency of IBD among populations across the world[14]. The incidence rate of PSC in the United States ranges from 0 to 0.92 per 100000 inhabitants per year, with no PSC patients being identified in Alaska between 1984 and 2000[4,15,16]. The prevalence of PSC in the United States is reported to be 13.6 per 100000 inhabitants[4]. Norway has the highest incidence rate at 1.31 per 100000 inhabitants and a prevalence of 8.5 per 100000[17]. The true prevalence of PSC may be higher than the aforementioned estimates both nationally and internationally because cholangiography may not be widely available in many parts of the world and patients with PSC may have normal levels of serum alkaline phosphatase (ALP)[18].

PSC can present itself at any age. The youngest individual documented to have PSC was under 2 years old[19]. UC is a major risk factor for the development of PSC, with approximately 60%-80% of patients having the PSC-UC phenotype[10,11]. However, only 4% of patients with UC have concomitant PSC[10,11].

PATHOGENESIS

The pathogenic mechanisms of PSC remain incompletely understood. Much like other autoimmune diseases, it has been theorized that the development of PSC is more likely to occur in a genetically susceptible individual after exposure to a trigger. PSC is the result of a complex immune-mediated response rather than a true autoimmune disease as it does not present with classic autoimmune features: female predominance, pathogenic autoantibodies, and response to immunosuppressive medications[20].

There is a 100-fold increased risk of developing PSC among siblings, however, the specific pattern of inheritance is much more complex[21,22].

Numerous studies have attempted to identify specific genes, which either predispose or protect an individual from the development of PSC. Many loci within the major histocompatibility complex have been linked to increased risk of PSC[23-25]. These are several class II HLA haplotypes including DRB1*0301-DRB3*0101-DQA1*0501-DQB1*0201, DRB1*1301-DRB3*0101-DQA1*0103-DQB1*0603, DRB1*1501-DRB5*0101-DQA1*0102- DQB1*0602, DRB1*0101-DQA1*0101, and B*0801[26-32].

HLA haplotypes associated with decreased risk of disease are DRB1*0401-DRB4*0103-DQA1*03-DQB1*0302, DRB1*0701-DQA1*0201-DQB1*0303, DRB4*0202-DRB1*1101-DQA1*0501-DQB1*0301, and MICA*002[29,31,32].

Due to the association of IBD with PSC, a "leaky gut" hypothesis has also been postulated[33]. Translocation of gastrointestinal (GI) flora from an inflamed GI tract to the portal venous system causes a systemic inflammatory response, which may disrupt the tight junctions in biliary epithelial cells[34,35]. This alteration exposes cholangiocytes to bile acids that could promote injury and inflammation[36].

Immune activation to an antigen (or a cross-reactive autoantigen or an enteric microbiome) also leads to inflammation of the gut and of the biliary tree[37]. Toll-like receptor and nucleotide oligomerization domain-like receptors assist in the detection of pathogens, which results in the secretion of pro-inflammatory cytokines[38]. Tumor necrosis factor (TNF)-α, transforming growth factor β1, interleukin (IL)-1β, and IL-6, along with involvement of CD8+ and CD4+ T cells

have been proposed to cause myofibroblast activation and fibrosis[38,39]. IL-2 has also been proposed as a key player in the regulation and programming of the immune system. IL-2 receptor α gene deficiency in mice causes biliary inflammation resembling PSC[40]. Integrin ligands, intracellular adhesions molecule 1, and vascular cell adhesion molecule 1 are also expressed by the biliary epithelium and contribute to the recruitment of inflammatory leukocytes that play a role in development of biliary inflammation seen in PSC[41]. Gut-specific T and B cells can be programmed to perpetuate biliary inflammation after encountering an enteric pathogen, providing an important rationale of how liver and gut inflammation may be linked[42].

A critical driver of disease development may be an altered biliary mucosal milieu, giving rise to biliary colonization of non-commensal bacteria[43]. Reduced biliary Proteobacteria and increased firmicutes due to a non-functional galactoside 2-α-L-fucosyltransferase 2 (FUT-2) enzyme appears to alter the commensal bacteria in the biliary tree[44].

The pathogenesis of PSC is likely the result of an amalgamation of a heightened immune response to a pathogen in a host with both an altered biliary mucosal milieu and a genetic predisposition to PSC.

DIAGNOSIS OF PSC

Signs and symptoms

Approximately 40%-50% of patients with PSC have no symptoms at initial presentation[3-5]. Among the symptomatic patients, fatigue, fever, jaundice, pruritus, and vague upper abdominal discomfort are most commonly described[3,6]. A sudden onset of jaundice, however, should prompt the clinician to inquire about an obstructive biliary process. As approximately 60%-70% of patients with PSC have coexisting UC, GI bleeding may also be seen in these patients.

Serologic markers

The hallmark of PSC is an elevation of alkaline phosphatase (ALP). ALP may vary throughout the course of disease and may also be normal in patients with PSC[45]. Improvement of serum ALP during the disease is a predictor of better outcomes and prolonged transplant-free survival[46]. Serum alanine (ALT) and aspartate (AST) aminotransferase levels may also be elevated to 2- to 3-fold above the upper limit of normal[10,47]. Serum bilirubin is usually normal at time of diagnosis of PSC, however, may be elevated in patients with advanced disease, malignancy, or superimposed choledocholithiasis[47,48].

Detectable autoantibodies are found in as many as 97% of patients with PSC[49]. The most commonly noted autoantibodies are anti-smooth muscle antibodies (ASMA) and antinuclear antibodies (ANA), which can be seen in up to 75% of patients[50]. Perinuclear antineutrophil cytoplasmic antibody and anti P-40 autoantibody can also be detected in approximately 30%-80% of patients with PSC and UC[51,52].

Proteinase-3 antineutrophil cytoplasmic antibody (PR3-ANCA) has been studied extensively for disease severity in patients with UC[53,54]. More recently, when measured using chemiluminescence immunoassay, PR3-ANCA was seen in 38.5% of patients with PSC compared to only 10.6% of patients with liver disease suggesting it is a better biomarker for the diagnosis of PSC[55]. Numerous other autoantibodies have been detected in patients with PSC, including anticardiolipin antibodies, thyroperoxidase, and rheumatoid factor[49]. These autoantibodies, however, are not routinely assessed for the diagnosis of PSC, as they may not be present in patients with PSC, and furthermore do not correlate with disease severity or disease prognosis.

Imaging

Cholangiography is considered the gold standard for the diagnosis of PSC. Historically, endoscopic retrograde cholangiopancreatography (ERCP) was the initial diagnostic procedure of choice, however, magnetic resonance cholangiopancreatography (MRCP) has become the preferred method of diagnosis of PSC in the past decade due to comparable specificity (> 90%) and sensitivity (80%-90%), associated lower cost, less invasive testing, and fewer complications[56-58]. The characteristic features include multifocal annular stricturing within intrahepatic and/or extrahepatic bile ducts, with alternating normal or slightly dilated segments of bile ducts (Figure 1), giving rise to the typical beads-on-a-string appearance[59]. Diffuse involvement of the hepatobiliary system may be seen, including stricturing of the gallbladder, cystic duct and pancreatic duct, however, approximately 25% of patients have isolated intrahepatic involvement[59]. Although MRCP is recommended as the initial imaging modality for the diagnosis of PSC, ERCP may be necessary in patients with a non-diagnostic MRCP or those who require therapeutic intervention for bile duct strictures.

Histologic features

A liver biopsy is rarely needed to confirm a diagnosis of PSC if characteristic cholangiographic findings are seen. Additional reasons why liver biopsies are not routinely obtained is the pathognomonic periductal fibrosis or "onion skinning" is not a common histologic finding in PSC[60]. Patients may have non-specific histologic findings and findings may be patchy and include more than one histological stage in at a given time indicating high sample variability[61].

When determining the stage of fibrosis, newer surrogate markers of cirrhosis including FibroSure (LabCorp) and transient ultrasound elastography (Echosens) have limited the need for a liver biopsy.

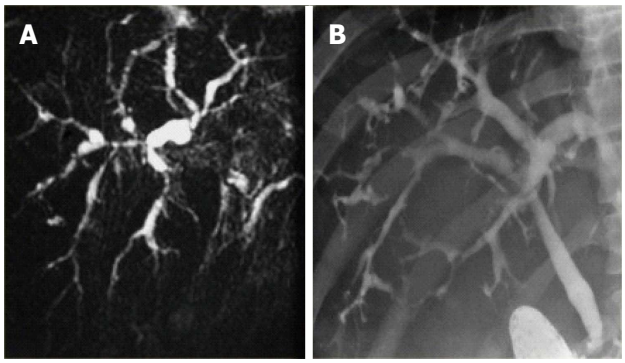

Figure 1 Imaging and endoscopy demonstrating primary sclerosing cholangitis. A: MRCP of a patient with PSC demonstrating intrahepatic stricturing with alternating normal and dilated segments of bile ducts; B: ERCP of a patient with PSC with similar findings. (Reproduced from Radiology Assistant. Levy AD, Chief of Gastrointestinal Radiology, Department of Radiologic Pathology, Armed Forces Institute of Pathology, Washington, D.C, United States). PSC: Primary sclerosing cholangitis; MRCP: Magnetic resonance cholangiopancreatography; ERCP: Endoscopic retrograde cholangiopancreatography.

VARIANT PSC SYNDROME

Small-duct PSC

Individuals with biochemical markers and histologic features suggestive of PSC with normal cholangiography are considered to have small-duct PSC[62]. Small-duct PSC represents a small proportion of patients with PSC and may even be an earlier stage of PSC[63]. This subgroup of PSC has been less studied than the classic large-duct PSC. Several studies suggest better prognosis in individuals with small-duct PSC, however, available data on this population is limited due to lack of long-term follow up[50,63,64]. A recent study with one of the longest follow-up of patients with small-duct PSC suggested that approximately a fourth of patients' progress to classic PSC in an average of 8 years[65]. Furthermore, individuals with small-duct PSC may progress to end-stage liver disease even without developing large duct disease and cholangiocarcinoma does not seem to occur in patients with small-duct disease in the absence of progression to large-duct PSC[65].

Lastly, the association of small-duct PSC appears to be stronger with Crohn's colitis than with UC[8,66].

Overlap syndrome (autoimmune hepatitis-PSC)

An overlap syndrome of PSC with autoimmune hepatitis (AIH) is more often diagnosed in younger adults, adolescents and in children[67,68]. Patients who exhibit features of both hepatocellular and cholestatic disease with the presence of ANA antibodies, the presence or absence of ASMA antibodies, and/or histological changes in the absence of AMA antibodies are considered to have autoimmune sclerosing cholangitis[69-71].

The diagnosis of AIH is based on the presence of characteristic clinical, laboratory and histologic findings, abnormal levels of serum globulins, and the presence of typical autoantibodies which ultimately provides the clinician with either a "definite" or a "probable" diagnosis of AIH based on the modified scoring system of AIH[72-74]. ANA and ASMA are typically seen in type 1 AIH whereas liver/ kidney microsomal type 1 antibody and liver cytosol type 1 antibody are observed in type 2 AIH[75]. Histologic lesions typically present in AIH are periportal lymphocytic or lymphoplasmacytic infiltration (interface hepatitis) with hepatocyte swelling[75]. In case of fulminant presentation, massive necrosis may also be present[75]. Treatment is largely based on immunosuppressive therapy with corticosteroids and azathioprine.

Many similarities exist between PSC and AIH, including the autoimmune serology, histologic findings, and the disease response to treatment with immunosuppressive agents. The International Autoimmune Hepatitis Group scoring system can help in the making a diagnosis of AIH, however, it is not recommended in making a diagnosis of AIH-PSC overlap syndrome[72]. In 2001, a cohort of 55 patients was evaluated to assess this overlap syndrome[67]. Approximately 50% of these patients had bile duct changes diagnostic of sclerosing cholangitis (SC) at the time of presentation and all but one of these patients would have been diagnosed with AIH type 1. Several other studies have also assessed this overall phenomenon in similar populations[19,76]. It is possible that the juvenile form of SC may represent an early stage of PSC in patients with concomitant AIH and progression to PSC is delayed with early use of immunosuppressive therapy.

IgG4-related Sclerosing cholangitis

A common manifestation of IgG4-related diseases (IgG4-RD) is IgG4-sclerosing cholangitis (ISC). Type 1 autoimmune pancreatitis (AIP) is the leading manifestation of IgG4-RD, affecting approximately 60% of patients with IgG4-RD, followed by sialadenitis affecting 34% of patients, followed by tubulointerstitial nephritis (23%), dacryoadenitis (23%), and periaortitis (20%)[77]. ISC is seen in approximately 20%-88% of patients with IgG4-RD[77-79]. Individuals with ISC are commonly diagnosed with concomitant AIP[80]. Similar to PSC and unlike classic autoimmune disease, ISC is more commonly seen in males with a male-to-female ratio of 4:1[77].

Analogous to PSC, patients typically present with vague abdominal pain. Individuals may also present with obstructive jaundice with concurrent AIP[79,81].

An elevated serum level of IgG4 is the most sensitive and specific method of diagnosing ISC, however, elevated levels of IgG4 may be seen in approximately 10% of patients with PSC, in approximately 15% of those with cholangiocarcinoma, and also in approximately 7% of patients who may have other ailments[82-84]. Several additional serologic

abnormalities may be seen in patients with ISC including elevated levels of IgG (approximately 60%), ANA positivity (approximately 40%), rheumatoid factor (approximately 20%), and IgE elevation (approximately 30%)[85-87].

Cholangiography may reveal multifocal biliary strictures, thickened bile duct wall and gallbladder wall thickening without vascular invasion[88].

Histologically, ISC demonstrates transmural fibro-inflammation with both fibrosis and inflammation evenly distributed from the mucosal surface to subserosa[89]. Immunostaining of the biopsy sample for IgG4 demonstrates diffuse infiltration of IgG4-positive plasma cells.

An approach for the diagnosis of ISC is the HISTORt criteria, which includes features on histology, imaging, serology, other organ involvement, and response to treatment with corticosteroids, and was initially utilized for the diagnosis of AIP and has been extended to include additional IgG4-related biliary diseases[78,90].

As the diagnostic algorithm suggests, rapid disease remission is achieved with immunosuppression using high-dose steroids[91]. Relapse of ISC can be seen in 30%-50% of patients and affected individuals may require re-induction of remission with additional high-dose steroids[91,92].

Long-term outcomes of patients with ISC are inconclusive and it remains unclear whether patients progress to end-stage liver disease or go on to develop cholangiocarcinoma.

PSC AND ASSOCIATED CONDITIONS

IBD and colorectal neoplasia

As previously mentioned, PSC has a strong association with IBD[7-9] with approximately 60%-70% of patients with PSC having coexisting UC, which often precedes the diagnosis of PSC or is diagnosed concomitantly[10,11]. Thus patients with PSC should undergo colonoscopic evaluation with biopsies despite the absence of typical symptoms[10]. If an initial evaluation does not reveal IBD, a repeat colonoscopy should be performed every 5 years to either confirm or exclude IBD[93].

Patients with IBD in the setting of PSC are considered to have a different phenotype (PSC-UC), which predicts a milder clinical course of the disease[9,94-96]. There also appears to be a right-sided predominance of diseased colonic mucosa, inflammation observed in the ileum and milder histologic inflammation in patients with PSC-IBD[97,98]. Due to the association of multiple malignancies with PSC, this disease entity should be considered a premalignant condition.

Patients with PSC-IBD have a significantly increased risk of developing colorectal malignancy compared to those with UC alone[99]. The cumulative CRC risk after 10 years of disease is 9% and 2%, which increases to 21%-30% and 5% in patients with PSC-IBD and isolated UC, respectively[99]. Moreover, colorectal cancer (CRC) and dysplasia are most often located in the right colon[95,96,98], which are associated with a worse prognosis when compared with left-sided colon cancer[100]. Annual or biennial surveillance colonoscopy is recommended in patients with PSC-IBD from the time of PSC diagnosis[101].

Cholangiocarcinoma

The most important risk is that of cholangiocarcinoma, which is several hundred times higher in patients with PSC than in patients without this disease[102]. Cholangiocarcinoma occurs in 1%-2% of patients annually following a diagnosis of PSC and is frequently detected within the first 1-3 years after the initial diagnosis[103,104].

Diagnosing cholangiocarcinoma in patients with PSC poses a tremendous challenge, as distinguishing between a benign dominant stricture from ductal cholangiocarcinoma requires the use of serologic, imaging, and ERCP over time. There are no designated risk stratification criteria, however, a commonly used approach involves annual MRCP or ultrasound examinations in conjunction with serum carbohydrate antigen 19-9 (CA19-9)[10]. If an individual is noted to have increasing serum levels of CA19-9, dominant strictures on imaging, and/or deterioration in either clinical status or liver test results, further assessment is made with an ERCP[105]. It is important to note that some individuals may not produce CA19-9 due to genetic reasons thus disease surveillance with this serologic test will not prove beneficial[106].

Routine brush cytology evaluation detects cholangiocarcinoma with low sensitivity (40%)[10] and near 100% specificity. Fluorescent *in situ* hybridization is used in conjunction with brush cytology specimens to apply a probe to subpopulations of cells with chromosome amplifications to assess for aneusomy. Individuals with the presence of a dominant stricture and polysomy [5 or more cells which have gained 2 or more chromosomes (3, 7, 17, and band 9p21)] are eventually diagnosed with cholangiocarcinoma with 88% specificity[107]. Cholangioscopy allows direct biliary visualization and directed biopsies of the dominant stricture and has been reported to have increased sensitivity and specificity to > 90%[108]. It is also being used to interrogate indeterminate strictures in an effort to enhance detection of cholangiocarcinoma. Confocal laser microscopy has not been studied in dominant strictures in PSC despite a high reported sensitivity and moderate specificity for indeterminate strictures in general[109]. Intraductal ultrasound may also offer improved diagnostic yield but is not widely adopted since its initial report for diagnosis of indeterminate strictures[110].

Gallbladder neoplasia

Concurrent abnormalities such as gallstone disease and PSC involving the gallbladder or cystic duct are seen

in approximately 41% of patients with PSC[111]. This population is also at an increased risk of developing gallbladder neoplasia, although the exact prevalence is unknown[112]. Although the malignant potential of gallbladder polyps smaller than 8 mm is small[113], the 2010 American Association for the Study of Liver Diseases (AASLD) guidelines recommend annual ultrasound and cholecystectomy if lesions are detected, regardless of the size[10]. Despite annual surveillance for gallbladder polyps, there is a lack of consensus regarding the malignancy potential of small polyps and surgical intervention in patients with advanced liver disease poses its own set of risks. It is important for the clinician to weigh the risks *vs* benefits of routine surveillance and surgical intervention.

TREATMENT OF PSC

Medical management

Historically, ursodeoxycholic acid (UDCA) has been used for the symptomatic improvement in cholestatic pruritus, which can be a debilitating consequence of PSC, and to improve abnormal liver chemistries. High dose UDCA (28-30 mg/kg per day) has been shown to increase the risk of colonic neoplasia in patients with PSC-IBD[114]. Additionally, there is a lack of definitive evidence that the use of moderate dose UDCA (15-20 mg/kg per day) improves survival in PSC patients or is efficacious in the prevention of colorectal cancer in those with PSC-IBD or biliary neoplasia[115,116]. Although the current AASLD guidelines recommend against the routine use of UDCA in patients with PSC, clinical practice varies between centers[10].

Azathioprine and steroids are recommended for use in patients with AIH as well as those with AIH-PSC overlap syndrome[10]. However, the use of immunosuppressive therapy (azathioprine, cyclosporine, tacrolimus, and methotrexate) and anti-TNF agent, infliximab, failed to demonstrate sustained improvement in abnormal liver chemistries and prevention of progression to end-stage liver disease in patients with PSC[117-120].

As newer investigations shine light on the link between biliary inflammation, bile acid homeostasis, and the gut microbiota, there is increasing interest in pharmacologic treatment of PSC with antimicrobial agents. The use of non-absorbable antibiotics, such as vancomycin, demonstrated improvement in liver chemistries in a small subset of patients[121]. An improvement in liver biochemistries was also observed with use of absorbable antimicrobials including metronidazole, azithromycin, and minocycline[122-124]. More data points are necessary to provide the clinician with definitive and accurate treatment options for PSC.

Novel treatment strategies, including the use of biologic therapy against lymphocytic trafficking in the pathogenesis of PSC, are being investigated. Vedolizumab (Millennium Pharmaceuticals, Takeda) is a gut-specific monoclonal antibody that selectively targets against $\alpha4\beta7$ heterodimer resulting in improvement in gut histology and mucosal T-cell infiltration[125]. It was approved by the Food and Drug Administration for induction and maintenance therapy for moderate to severe UC and Crohn's disease in 2014[126,127]. Its use in patients with PSC-IBD is theorized to take effect by the presence of gut adhesion molecules and the entero-hepatic expression in PSC, however, the clinical utility of vedolizumab in PSC-IBD patients remains under investigation[37,128].

Management of biliary strictures

Patients with worsening symptoms over the disease course require investigation to exclude the presence of an extrahepatic dominant biliary stricture. A dominant stricture is defined as an area of stenosis $\leqslant$ 1.5 mm in the common bile duct or $\leqslant$ 1 mm in the common hepatic duct and is present in approximately 50% of PSC patients[10,129].

Dominant strictures are treated by either dilation alone or with dilation and placement of temporary plastic biliary stents during the ERCP[130]. It is important to note that PSC patients undergoing ERCP should be provided with prophylactic antibiotics to prevent possible cholangitis[131]. The duration of endoscopic therapy is variable and can range from 6 weeks to 12 mo before strictures resolve. ERCP with repeated stenting may be required in some patients who are refractory to dilation[130]. Due to the risk of cholangiocarcinoma masquerading as a dominant stricture, brush cytology and/or biopsy samples should be obtained during the endoscopic procedure[132]. The utility of ERCP is solely for the exclusion of cholangiocarcinoma and to provide therapy for dominant biliary strictures and does not modify the progression of the disease[130].

Liver transplantation

Due to the lack of durable pharmacologic and endoscopic therapy, liver transplantation (LT) remains the sole curative option in patients with progressive disease. PSC is the fifth most frequent indication for LT in the United States[133]. Intractable pruritus, recurrent bacterial cholangitis, and perihilar cholangiocarcinoma are additional indications for LT in PSC patients.

Post-transplant acute rejection can be seen within the first 30 d of transplantation, but usually resolves with systemic corticosteroids and does not appear to alter graft survival[134].

In patients with cholangiocarcinoma, LT in conjunction with neoadjuvant chemotherapy and radiation should be considered[135,136].

Patients with PSC-IBD may develop worsening disease post LT and approximately 14%-30% of patients with PSC may go on to develop de novo IBD up to 10 years post LT[10]. Patients should be monitored with serial serum ALP measurements post

LT as increasing levels indicate recurrence of disease. Recurrent PSC, despite LT, is seen in 30%-50% of patients within 10 years of transplantation[137]. To date, no medical therapy has been identified to halt the progression or recurrence of disease.

CONCLUSION

PSC is a fascinating and largely elusive entity within the realm of hepatobiliary diseases. This is a chronic cholestatic liver disease, which has a tremendous impact on survival of those who are affected. Over time, many treatment modalities have been evaluated, however only LT is a known therapeutic option in these patients. Although newer drugs continue to be investigated for the treatment of PSC, effective treatment options remain limited. Future research in genomic-based therapies will hopefully allow for alteration of the natural course of this disease.

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REVIEW

Biliary tract cancer stem cells - translational options and challenges

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Abstract

Management of biliary tract cancer remains challenging. Tumors show high recurrence rates and therapeutic resistance, leading to dismal prognosis and short survival. The cancer stem cell model states that a tumor is a heterogeneous conglomerate of cells, in which a certain subpopulation of cells - the cancer stem cells - possesses stem cell properties. Cancer

stem cells have high clinical relevance due to their potential contributions to development, progression and aggressiveness as well as recurrence and metastasis of malignant tumors. Consequently, reliable identification of as well as pharmacological intervention with cancer stem cells is an intensively investigated and promising research field. The involvement of cancer stem cells in biliary tract cancer is likely as a number of studies demonstrated their existence and the obvious clinical relevance of several established cancer stem cell markers in biliary tract cancer models and tissues. In the present article, we review and discuss the currently available literature addressing the role of putative cancer stem cells in biliary tract cancer as well as the connection between known contributors of biliary tract tumorigenesis such as oncogenic signaling pathways, micro-RNAs and the tumor microenvironment with cancer stem cells.

Key words: Biliary tract cancer; cancer stem cells; Cancer stem cell markers; Tumor microenvironment; Micro-RNAs

Core tip: Using a xenograft model, researchers successfully demonstrated that as few as ten of a specific subpopulation of biliary tract cancer cells had the potency to (serially) establish and recapitulate biliary tract cancer in immunodeficient mice. Furthermore, expression of established cancer stem cell markers, cancer stem cell-related signaling pathways and micro-RNAs was reported in biliary specimens and cell lines - in most cases associated with clinical outcome. Based on these results, the existence of cancer stem cells in biliary tract is well-founded and potentially harbors new options for development of therapeutic strategies.

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INTRODUCTION

General aspects of cancer stem cells

In 1994, Lapidot *et al*[1] identified a subpopulation of cells, characterized by a specific set of surface markers that was able to initiate acute myeloid leukemia in mice. Since then, numerous of such tumor-initiating cells were identified in most solid tumors[2]. These tumor-initiating cells, also referred to as cancer stem cells (TICs or CSCs) have the ability to self-renew as well as to differentiate into different lineages - traits that they share with (adult) stem cells. Further similarities between tumor-initiating cells and normal adult stem cells include the reliance on certain highly conserved embryonic pathways (such as Hedgehog (Hh), Nanog and Wnt), a specialized metabolism (preferential oxidative glycolysis), enhanced protection against DNA damage and oxidative stress, a specific epigenetic profile (*e.g.,* abnormal polycomb repressive complexes activity, see below) as well as the expression of specific surface markers (reviewed in[2]). The specific abilities and profiles of CSC may at least in part explain some of the common clinical problems seen when dealing with cancer. CSCs are slow-cycling cells that often are in a quiescent state. Common chemotherapeutics target proliferating, fast cycling cells, thereby erasing the bulk of the tumor while not affecting CSCs - a phenomenon resulting in tumor recurrence. Moreover, CSCs strongly express drug efflux pumps, contributing to the well-known chemoresistance of these cells[2-5].

Currently, two main models are discussed regarding the origin of CSCs[6]. In the "stochastic model", each cancer cell is biologically equivalent and unpredictably may acquire a CSC phenotype depending on diverse stochastic events from inside the cells (*via* genetic and epigenetic changes) as well as from the surrounding environment. The second model called "hierarchic model" states that a tumor is, like solid organs, a hierarchically organized heterogeneous cell conglomerate in which only a small subset of cells - the CSC - have the ability to self-renew and to give rise to daughter cells of various differentiation, whereas the majority of cancer cells that form the bulk of the tumor cannot achieve CSC traits. Besides these two main models, a third possible origin of CSC is currently discussed in the literature, namely de-differentiation of already committed cells - a phenomenon that was observed in different tumor entities and that is likely to play a role in biliary tract cancer (BTC) as discussed later in this article[7-10].

EXPERIMENTAL IDENTIFICATION AND CHARACTERIZATION OF CANCER STEM CELLS

Identification and/or isolation of CSC based on their expression profile (surface markers, signaling pathways) as well as their functional characteristics represent powerful tools in cancer research. The ability of CSCs to form tumors in immunodeficient mice at very low cell numbers surrogates their high tumorigenic potential[2]. Besides *in vivo* xenograft experiments, also several *in vitro* techniques are used in CSC research. The clonogenic assay similarly addresses the higher tumorigenic potential of CSC. Here, very few cells (approximate range between 50-200 cells per cm^2 - highly cell line-dependent) are seeded in a cell culture receptacle and the tumorigenic potential is evaluated by counting the number of growing colonies, each of them originating from a single cell clone representing

a potential CSC[11]. Anchorage-independent growth, *i.e.,* the formation of tumor spheres in a non-adherent environment, is another well-established experimental approach used in CSC research[12]. Likewise, expression of aldehyde-dehydrogenase 1 (ALDH1) is considered as a functional CSC marker[13]. ALDH1 is a detoxifying enzyme that was shown to be up-regulated in cancer and associated with various CSC traits such as enhanced tumor growth and the potential of self-renewal and differentiation[13-16]. Moreover, the so-called side-population phenotype may signify a CSC population. Side-population cells are defined by their ability to excrete fluorescent dyes such as Hoechst 33342 - this characteristic is based on the enhanced expression of efflux pumps in (cancer) stem cells[17,18]. Lastly, potential CSCs are identified and isolated based on their surface markers using fluorescence-activated cell sorting or by analysis of expression of established (cancer) stem cell genes.

BILIARY TRACT CANCER - CLINICAL BACKGROUND

BTC is a heterogeneous malignancy that arises from different locations within the biliary tree. It can be categorized into intrahepatic cholangiocarcinoma (IHC), extrahepatic cholangiocarcinoma (EHC), gallbladder cancer (GBC) and mixed hepatocellular-cholangiocarcinoma (HCC-CC). Although BTC is generally a rare disease, it has high clinical significance due to its dismal outcome and limited therapeutic options[19,20]. Due to late diagnosis, for most patients only palliative treatment is possible; furthermore, the standard chemotherapeutic approach using a combination of cisplatin and gemcitabine results in median survival of approximately one year only[21]. Therefore, advances in understanding the underlying mechanisms of BTC development, progression and aggressiveness are of utmost importance for better management of this disease. BTC is characterized by high recurrence rates, formation of metastasis and high therapeutic resistance towards conventional chemotherapy regimens[19,20]. The involvement of CSC subpopulations in BTC is likely, however, the current literature is sparse. In this article, we discuss current studies on the role and impact of CSCs in BTC. Specifically, we focus on potential CSC markers and signaling pathways in BTC and the clinical consequences of their expression as well as on giving an overview of other aspects of BTC tumorigenesis such as miR expression and tumor microenvironment that can be linked to BTC CSCs.

POTENTIAL ORIGINS OF BILIARY TRACT CANCER CELLS WITH STEM CELL-LIKE CHARACTER

Several hepatic cell types have been suggested to represent the origin of BTC CSC (summarized in Figure 1)[22]. Mature hepatocytes have the ability to de-differentiate into more pluripotent cells through mechanisms of cell plasticity and reprogramming, thereby acting as a population with stem cell traits[23]. IHC is categorized as a primary liver tumor and shows characteristics of both hepatocellular carcinoma and cholangiocarcinoma, suggesting a potential link regarding the cell of origin[24,25]. Evidence for mature hepatocytes being the source and quasi-CSC for IHC comes from two studies that showed that mature hepatocytes are able to transdifferentiate and form IHC[9,10]. In another study, it was shown that loss of tumor suppressor p53 contributes to dedifferentiation of hepatocytes into progenitor cells that can transform into IHC[26]. This is especially interesting, since loss of p53 is a major genetic characteristic of BTC[27]. Cells residing in the Canals of Hering, a hepatic stem cell niche, are another possible source of BTC-initiating cells[28]. Because the Canals of Hering represent the interface between the liver and the biliary system, residing stem cell populations may be (1) hepatic progenitor cells; (2) biliary progenitor cells; or (3) bi-potential progenitor cells, called hepatoblasts in humans and "oval cells" in rodents which have the ability to differentiate into hepatocytes and cholangiocytes[25,29-31]. There is evidence that IHC can originate from hepatic stem cells. Expression of α-fetoprotein (AFP), a protein expressed by fetal hepatocytes and hepatic progenitor cells, was demonstrated in IHC[32-34]. In addition, AFP expression was shown in BTC cell lines and these AFP-expressing BTC cells had characteristics of stem cells[35]. Of note, IHC subtypes that were identified to have a hepatic stem-like gene signature had very poor prognosis, underlining not only the connection between hepatic stem cells and IHC, but also the clinical relevance and consequences[36]. The peribiliary glands were also described as a source of biliary stem cells as they are involved in normal biliary tissue turnover and repair[37,38]. BTC often occurs under (chronic) inflammatory conditions and it was shown that during primary sclerosing cholangitis, a chronic inflammation of the bile ducts, biliary tree stem cells are activated in the peribiliary glands[39]. It can be speculated that due to the chronic inflammation and concomitant constant activation of these normally quiescent stem cells, under these tumor-promoting conditions these cells become exposed to an environment that potentially causes malignant transformation - which is especially relevant taking into account the intrinsic longevity of these cells allowing for accumulation of malignant events which eventually may lead to a tumorigenic CSC phenotype. Moreover, p63, which is a homologue of p53 and also a stem cell marker of prostate gland and squamous cells was aberrantly expressed in IHC arisen from cirrhotic liver[40-42], underlining the connection between (chronic) inflammation and CSC in BTC.

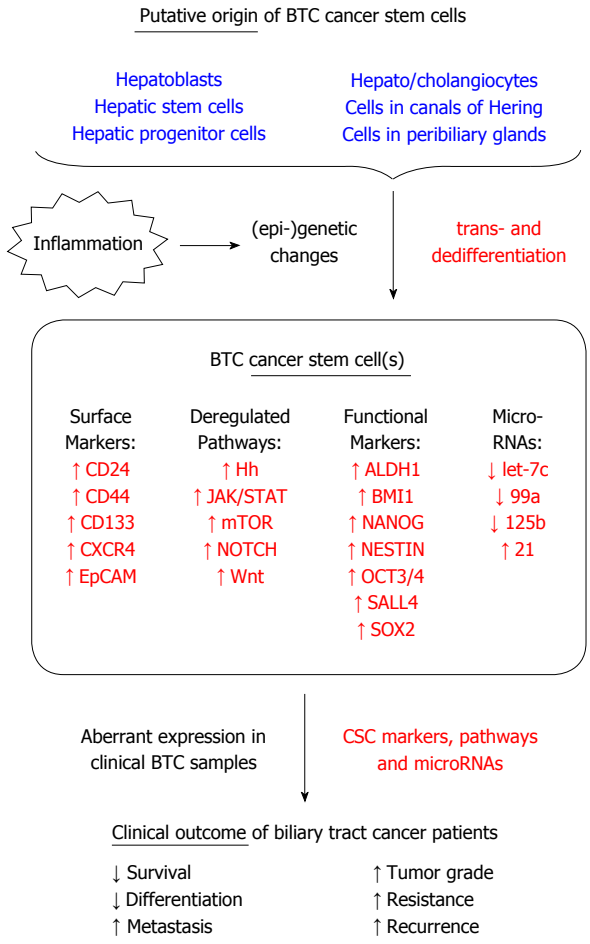

Figure 1 Cancer stem cells in biliary tract cancer. Biliary tract cancer stem cells are thought to originate from various subpopulations of healthy cells that harbor stem cell or stem cell-like traits. Currently available data on clinical biliary tract cancer (BTC) specimens revealed up-regulation of established cancer stem cells (CSC) cell surface and functional markers as well as aberrant activity of signaling pathways and micro-RNA species. Expression of CSC markers and stemness factors in BTC tissues is associated with diverse unfavorable clinico-pathological features and poor prognosis. See text for details.

In general, these studies suggest that there may not be one BTC CSC population but rather several different CSC populations, which in turn mirrors the heterogeneity of BTC and makes targeted and personalized (CSC-based) therapy very challenging and a demanding aim in the future.

CANCER STEM CELL MARKERS IN BILIARY TRACT CANCER - AN OVERVIEW

Limiting dilution cell transplantation assay in immunodeficient mice is a method to determine tumorigenic potential of cancer cells. CSCs, by their nature, harbor high tumorigenic potential, meaning that only few of these cells are able to form a heterogeneous tumor. Raggi *et al*[43] isolated BTC cells with sphere formation potential from parental BTC cells and demonstrated that these cells retained their potential to form spheres over several passages, indicating self-renewal potential. Furthermore, when injected into immunodeficient mice, as few as ten of these cells were able to generate tumors and this high tumorigenic ability was retained when re-transplanted[43]. Gene expression analysis of these potential BTC CSCs revealed up-regulation of a number of genes including genes responsible for self-renewal and pluripotency (*e.g.*, SOX2, BMI1, NOTCH1), drug resistance (*e.g.*, ABCG2), surface markers (*e.g.*, CD24, CD44, EpCAM) and metastasis[43] that are also expressed in regular stem cell populations. Another study by Wang *et al*[44] further supports the existence of CSCs in BTC: they demonstrated in xenograft experiments that the subpopulation of CD24+/CD44+/EpCAM^{high} cells harbor high tumorigenic potential compared to the CD24-/CD44-/EpCAM^{low} counterparts. Furthermore, they showed via serial *in vivo* passaging, that the expression profile of CD24/CD44/EpCAM remained stable comparable with the primary tumor. In addition, tumors resulting from injection of CD24+/CD44+/EpCAM^{high} cells contained both, CD24+/CD44+/EpCAM^{high} as well as phenotypically different cell populations, demonstrating the ability of CD24+/CD44+/EpCAM^{high} to self-renew and to produce heterogeneous daughter cell populations[44].

Expression of several of these established surface and general CSC markers was identified in BTC specimens and cells. As shown in Table 1, expression of these markers was generally associated with disadvantageous clinico-pathological characteristics and shorter disease-free and overall survival. In addition, several *in vitro* studies investigated downstream targets and processes that are directly connected with the expression of these CSC markers in BTC. Resistance to anti-tumor treatments is a hallmark of cancer and CSCs and caused by up-regulation of genes responsible for drug efflux and DNA repair[4]. Nakashima and colleagues demonstrated an increase of proportion of CD24+/CD44+ cells in gemcitabine-resistant BTC cells and showed that genes of the BRCA/Fanconi repair pathway was over-expressed here, thus connecting the observed chemoresistance in these CSCs with a particular repair pathway[45]. Expression of the drug efflux pump ABCG2 is another mechanism of cells to gain therapeutic resistance and also an established CSC marker[46]. In BTC, ABCG2 was shown to be over-expressed in BTC tumor spheres and in CD44+/CD133+ cells, making it a candidate for pharmacological intervention in putative BTC CSCs[43,47,48].

Chemokine receptor 4 (CXCR4) plays an important role in repair and regeneration of tissue in adults and was also identified as a surface marker of (cancer) stem cells[49]. Using a comprehensive gene analysis array, Leelawat *et al*[50] compared the expression profile of CD24+ and CD24- BTC cells and found enhanced expression of CXCR4 in the CD24+

Table 1 Surface and functional cancer stem cell markers in biliary tract cancer and their clinical consequences

Marker	Tissue	M	R	S	TS	Comment	Ref
Surface stem cell markers							
CD24	CC, IHC	↑	↑	↓			[68-70]
CD44	CC, EHC, IHC, peri-hilar CC, HCC-CC	↑		↓		↓differentiation, ↑recurrence	[28,71-75]
CD133	CC			↓	↑		[28,63,71]
CXCR4	GBC, IHC	↑		↓	↑	↑vascular invasion	[52,76]
EpCAM	IHC			↓			[28,77]
Functional stem cell markers							
ALDH1	EHC, IHC, perihilar CC		↑	↓			[58,78]
BMI1	EHC, HCC-CC, IHC, perihilar CC					↑in tumor specimens	[71,79,80]
NANOG	EHC, IHC, perihilar CC						
NESTIN	EHC, IHC, perihilar CC						
OCT3/4	GBC, CC	↑		↓	↑	↑tumor size	[63,81]
SALL4	IHC	↑		↓		↑vascular and nerve invasion	[82]
SOX2	EHC, IHC, perihilar CC	↑		↓	↑		[71,74]

CC: Cholangiocarcinoma; EHC: Extrahepatic cholangiocarcinoma; GBC: Gallbladder cancer; HCC-CC: Combined hepatocellular-cholangiocarcinoma; IHC: Intrahepatic cholangiocarcinoma; M: Metastasis; R: Therapeutic resistance; S: Survival; TS: Tumor stage.

subpopulation. Drug-based inhibition of CXCR4 using AMD3100 suppressed migration and invasion of BTC cells, and this effect was only observable in the CD24+ CSC subpopulation[50]. Interestingly, AMD3100 treatment also reduced sphere formation potential of BTC cells in another study, further connecting CXCR4 expression with stem cell characteristics[51]. RNA interference-mediated knockdown of CXCR4 in an IHC model inhibited proliferation and colony formation *in vitro* as well as tumorigenicity *in vivo*[52].

Cardinale *et al*[53] demonstrated in xenograft experiments that CD13+/CD90+ BTC spheroids were highly tumorigenic. Interestingly, these two surface molecules are established CSC markers that also play a role in liver cancer, further confirming that hepatocellular carcinoma and BTC may share a common origin or CSC subpopulation[54-57].

As mentioned above, CSC can also be identified via functional characteristics such as ALDH1 expression[13]. Using a BTC cell model, Shuang *et al*[58] demonstrated that, in contrast to ALDH- cells, ALDH+ cells were able to form tumor spheres. In addition, epithelial markers were reduced in ALDH+ cells, whereas mesenchymal markers were strongly expressed - connecting this cell population to epithelial-to-mesenchymal transition (EMT), a process that is closely related to CSC as discussed later in this article[58,59].

It is well established that deregulated epigenetic events play a huge role in transformation of cells towards a malignant phenotype. The polycomb repressive complexes (PRC) 1 and 2 are multi-protein epigenetic regulators which are known to be aberrantly active in cancer and essential for CSC to maintain their stemness character[60]. Several studies indicate a pivotal role of PRC1 and 2 in development and progression of BTC (as reviewed in[61]). BMI1, which was shown to be expressed in BTC patient samples (Table 1), is a core component of the PRC1 and recently it was demonstrated in BTC cells, that pharmacological inhibition of BMI1 resulted in reduction of ALDH+ cells and diminished formation of tumor spheres[62]. Moreover, in another study, BMI1 was found to be significantly higher expressed in BTC cells positive for CD133 and OCT3/4, further suggesting BMI1 as a CSC marker in BTC[63].

Regarding the pluripotency markers NANOG, OCT3/4 and SOX2[64], only few studies investigated their expression and associated outcomes in clinical BTC samples. However, several *in vitro* and in *vivo* studies suggest a pivotal role of these pluripotency markers in BTC CSC. In CD133+ spheres derived from GBC cells, OCT4 and NANOG were highly expressed and these cells also showed higher resistance to chemotherapeutics[65]. In line with these findings, two other studies found that in tumor spheres derived from BTC cells, stem cell markers such as CD133, NANOG, SOX2, SALL4 and OCT4 were up-regulated[53,66]. In addition, these spheres over-expressed ABCG2 and were resistant to cisplatin and additionally displayed high tumorigenic potential when injected into nude mice[66]. More evidence for SOX2 being a potentially relevant factor in BTC cells with stem cell character was presented in another study where the authors showed that artificially over-expression of SOX2 enhanced proliferative capacity, apoptosis resistance and migration and invasion potential[67].

RELEVANCE OF STEMNESS PATHWAYS IN BILIARY TRACT CANCER STEM CELLS

Several signaling pathways are involved in generation and maintenance of CSCs, including the embryonic signaling cascades NOTCH, Wnt and Hh as well as the interleukin-6 (IL-6)-JAK/STAT cascade and the mTOR pathway (for detailed pathway descriptions see[5,83,84]). Of note, these embryonic signaling pathways are also involved in basic cholangiocyte differentiation[85].

Several studies found deregulation of these signaling pathways in BTC specimens and corres-

Table 2 Overly active pathways associated with cancer stem cell-like phenotype in biliary tract cancer specimens

Pathway	Component	Tissue	Outcome	Ref.
Hh	GLI1	GBC	↑ lymph node metastasis	[92,100]
	SHH	CC, GBC	↑ grade (by trend)	[92,100,101]
	SMO	GBC		[92]
JAK / STAT	STAT3	CC, IHC	↑ tumor size, ↑ metastasis, ↑ vascular invasion, ↓ survival, poor histological differentiation	[102,103]
mTOR	mTOR	BTC	↓ survival	[104]
	pmTOR[1]	GBC	↓ survival	[105]
NOTCH	NOTCH 1	EHC	poor histological differentiation, ↓ survival, ↑ tumor grade, ↑ Cyclin E	[86,87]
	NOTCH 2	EHC	↓ survival	[86]
	NOTCH 3	EHC	↓ survival, ↑ tumor grade, ↑ Cyclin E	[86,87]
	NOTCH 4	EHC		[86]
	HES-1	EHC	↓ survival	[86]
Wnt	β-catenin	CC	↑ metastasis	[91][2]

[1]Phosphorylated mTor; [2]In this study, no non-tumor control samples were used as control. CC: Cholangiocarcinoma; EHC: Extrahepatic cholangiocarcinoma; GBC: Gallbladder cancer; IHC: Intrahepatic cholangiocarcinoma.

ponding poor clinical outcome parameters (Table 2 and Figure 1). In addition to these findings, in depth experimental approaches as well as experiments on pharmacological intervention of these pathways have shed more light on their roles in putative CSC in BTC. Treatment of cells with the NOTCH pathway inhibitor γ-secretase inhibitor (GSI) IX significantly decreased the CD24+/CD44+ subpopulation in a BTC cell line model[86]. Moreover, single treatment with gemcitabine increased the amount of CD24+/CD44+ BTC cells, whereas combined treatment with gemcitabine and GSI IX mitigated this effect[86]. In an interesting study by Zender *et al*[87] long-term artificial over-expression of Notch Intracellular Domain (NICD) 1 - an integral factor of the NOTCH signaling cascade - in mouse livers resulted in a cell population that, when injected into immunodeficient mice, was able to form BTC with features of hepatic progenitor cells. This not only demonstrates the involvement of the NOTCH signaling pathway in CSC, but also suggests a role of hepatic progenitor cells in the development of BTC. In the same regard, Ishii and coworkers published that NICD1 was expressed exclusively in BTC cells with CSC characteristics. Furthermore, treatment with GSI DAPT decreased the number of potential BTC CSCs[35]. Combined drug-based Hh and mTOR inhibition reduced viability and proliferation of BTC cells *in vitro*[88]. Intriguingly, combined treatment also reduced the number of ALDH+ cells as well as the expression of pluripotency factors NANOG and OCT4. Additional *in vivo* experiments also revealed diminished tumorigenic potential of the treated cells, indicating concerted action of the Hh and mTOR pathway in creation and /or maintenance of a CSC phenotype in BTC[88]. Constitutively β-catenin expression (mimicking active Wnt pathway) promoted self-renewal of hepatic progenitor cells and injection of these cells generated tumors with characteristics of HCC-CC[89].

Cellular plasticity of differentiation is discussed as a characteristic of CSC in BTC[90]. A phenomenon tightly connected to cellular plasticity and therefore to a CSC phenotype is the ability of cells to detach from the primary tumor and to gain access to the lymphatic and/or vascular system, *i.e.,* the cells become invasive. In order to do so, these invasive and potentially stem cell-like cancer cells have to carve through the surrounding extracellular matrix. Matrix metallopeptidases (MMP) are enzymes that are centrally involved in the breakdown of the extracellular matrix. Active Wnt pathway was shown to directly up-regulate MMP expression in BTC cells, giving BTC cells the ability to gain invasive capabilities[91]. More evidence that the Wnt pathway influences the CSC phenotype in BTC comes from Zhao and coworkers: knockdown of the BTC CSC marker CXCR4 (Table 1) also caused inhibition of the Wnt pathway[52]. Direct causality of these events was proven by providing the ligand CXCL12, which resulted in activation the Wnt pathway and the respective downstream targets including CD44[52].

Artificial activation of Hh pathway (*via* up-regulation of the ligand SHH) enhanced invasiveness of GBC cells and this effect was reversible via shRNA-mediated or drug-based (cyclopamine) intervention[92]. Further experiments showed that regulation of MMP expression via the Hh pathway was a potential underlying molecular mechanism of this observation[92]. In the same study, Hh pathway activation was associated with enhanced colony formation and this effect was reversed by RNA interference-mediated Hh pathway blockage. Finally, GBC cells with active Hh signaling harbored greater tumor-generating capability *in vivo*, underlining the importance of this pathway in BTC for several aspects connected with CSC characteristics[92].

BTC often develops under inflammatory conditions and in this regard, diverse cytokines such as IL-6 are involved. It was shown that under chronic inflammatory conditions, neoplastic cholangiocytes are able to produce and secrete IL-6 in an autocrine loop, resulting in proliferation and induction of DNA damaging molecules such as reactive oxygen species and nitric oxide[24]. The JAK/STAT signaling

cascade is one of the downstream pathways that is activated via inflammation-related cytokines in BTC development[93,94]. In an interesting study using GBC cells, Kong *et al*[95] isolated side-population cells and compared the functional and molecular characteristics of these potential CSCs with the non-side-population cells. They found enhanced expression of IL-6 and activated (*i.e.,* phosphorylated) STAT3 in the side-population cells. Intriguingly, these cells harbored multiple CSC traits: enhanced tumor sphere and colony formation potential, chemoresistance, the ability to generate both, side-population and non-side-population cells and, finally, high tumorigenic potential *in vivo*[95]. Drug-based inhibition of STAT3 reduced growth and migration/wound healing potential of BTC cells[94]. Moreover, treatment of cells with the natural compound luteolin suppressed the activation of the IL-6-induced JAK/STAT3 cascade in BTC cells which resulted in diminished migration, wound healing and colony formation potential[96].

mTOR inhibition was directly connected to loss of CSC characteristics in BTC cell lines: treatment with rapamycin decreased migration, invasion as well as sphere formation potential[97]. Similar results were presented by two other studies in GBC cells[98,99]. Of note, activated mTOR pathway was found in highly proliferative and metastatic GBC cells[99].

MIRNAS AND THEIR ROLE IN REGULATION OF BILIARY TRACT CANCER STEM CELL CHARACTERISTICS

Micro-RNAs (miRs) are non-coding RNAs that regulate gene expression by binding and degradation of target mRNAs[106]. As recently reviewed, aberrant miR expression is involved in different aspects of BTC development and progression[107]. However, up to now, only few studies associated deregulated miR expression with CSC characteristics in BTC. The miRs let-7c/99a and 125b originate from the same gene cluster and were shown to be down-regulated in CC patient material, de-facto being tumor suppressor miRs[108]. Interestingly, expression of these miRs was reduced in tumor spheres derived from BTC cells and in addition, enforced expression of let-7c/99a and 125b reduced the expression of CD133 and CD44 in BTC cells as well as the potential to form tumor spheres[108]. Of note, in the same study, the Interleukin 6 (IL-6) pathway, including IL-6 itself, its receptor IL-6R and the downstream transcription factor STAT3 were identified as targets of this particular miR cluster.

MiR21 is a potent oncogenic miR in BTC with several established targets that contributes to disadvantageous clinical outcome[107]. Zhang *et al*[109] published that miR21 is necessary for survival of CD24+ cells in primary liver cancer, indicating a role of miR21 in the CSC phenotype in BTC.

Although the number of studies regarding miRs and CSC in BTC is limited as of today, the overlap between described deregulated miRs in BTC and the role of these miRs in CSC of other tumor types strongly suggests a role of these miRs also in CSC of BTC. For example, miR200b is down-regulated in BTC samples, resulting in shorter survival[110]. In lung adenocarcinoma, reduced miR200b expression was shown to be a marker of CSC and, interestingly, restoration of miR200b expression resulted in loss of CSC maintenance and chemoresistance[111]. MiR145 is another miR species that was found to be down-regulated in BTC specimens and associated with poor survival[112]. Likewise, in lung adenocarcinoma tissues, miR145 was found to be down-regulated and negatively correlated with expression of OCT4[113]. Moreover, forced expression of miR145 in lung cancer initiating cells markedly reduced CSC features *in vitro* and *in vivo*[113]. In prostate cancer, miR34a was down-regulated in the CD44+ CSC subpopulation and the down-regulation of this miR species was also measured in BTC samples and correlated with advanced clinical stage, lymph node metastasis and poor survival[114,115].

BRIEF OUTLOOK ON BILIARY TRACT CANCER STEM CELLS AND TUMOR MICROENVIRONMENT

CSCs are thought to reside in specific environments called stem cell niches. The CSC niche consists of various cell types and structures including immune cells, mesenchymal (stem) cells, fibroblasts, vascular network, soluble factors and extracellular matrix components and has the function to preserve the exclusive features of CSC as well as to protect them from therapeutic intervention[116]. By creating a suitable tumor microenvironment (TME), the CSC niche plays an outstanding role in development and progression of cancer, essentially supporting tumor growth in multiple aspects. On the other hand, CSCs also support their TME for example by inducing the expression of survival genes[116]. Data regarding CSC niches and TME in BTC are very limited, however, the TME likely contributes to angiogenesis, invasion, metastasis, therapeutic resistance, maintenance of CSC niche and survival of CSC also in BTC[117]. In an interesting study, Raggi *et al*[43] demonstrated the importance of the interaction between BTC cells and macrophages for tumor development. Medium gathered from BTC cells with CSC characteristics (spheres) activated CD14+ macrophages and shifted their phenotype towards CD163+ so-called tumor-associated macrophages (TAM) which harbored high invasive capacity accompanied by expression of the matrix-remodeling gene MMP2. In the same study, CD163+ macrophages were found at the tumor front in BTC samples, suggesting the importance of these immune cells in progression of BTC. Moreover, the levels of TAMs were associated with poor prognosis of

BTC patients[118-120]. Evidence that BTC CSCs not only are able to activate TAMs but also to chemo-attract them was shown *via* chemotaxis experiments, in which CD14+ strongly migrated towards medium derived from BTC spheres[43].

Mesenchymal stem cells (MSCs) are multipotent adult stromal stem cells that have the ability to generate diverse types of connective tissue[121]. They produce a wide range of cytokines and chemokines, thereby strongly communicating with their near and far environment. Several studies demonstrated that MSCs not only support tumor growth and CSC as a part of the TME, but also that MSC actively migrate towards tumor sites[122]. Regarding BTC, media from MSCs increased migration, invasion, proliferation, chemoresistance and colony formation potential of BTC cells *in vitro*[91]. Moreover, media from MSCs activated Wnt/β-catenin signaling as well as MMP expression. In a xenograft model, the authors also demonstrated that injection of BTC cells together with MSCs resulted in significantly larger tumors[91]. Recently, it was shown that media derived from BTC cells enhanced the migratory potential of MSC as well as the release of IL-6[123]. On the other hand, culture media derived from these "activated" MSCs significantly increased proliferation of BTC cells as well as the amount of pSTAT3. This effect was completely blocked by the addition of an anti-IL-6 antibody. This is especially interesting because of the well-known role of the IL-6/JAK/STAT cascade in CSCs (see above), thus indicating that MSC can directly support CSCs in BTC. Further evidence that MSCs may directly interact with CSC comes from the fact that MSCs secrete CXCL12, the ligand of the putative BTC stem cell surface marker CXCR-4 (Table 1)[121].

Epithelial-to-mesenchymal transition (EMT) is a process in which cells lose their epithelial traits and gain mesenchymal character, allowing them to detach from the primary tumor and subsequently to form secondary tumors. On molecular level, loss of epithelial markers such as E-Cadherin and enhanced expression of mesenchymal markers such as Vimentin can be observed during EMT[124,125]. Key factors that can initiate EMT include Slug, Snail and Twist, which are repressors of E-Cadherin[125]. The process of EMT is closely related to CSC phenotype. For example, several established CSC pathways such as Wnt, NOTCH and mTOR are involved in EMT and forced expression of EMT resulted in enrichment of CSC subpopulation (as reviewed in[126]). For BTC, Shuang *et al*[58] connected an EMT phenotype with CSC characteristics. They demonstrated that the ALDH+ subpopulation expressed low levels of E-Cadherin and high levels of the mesenchymal markers Vimentin and N-Cadherin. In another study, the authors recognized reduced expression of the ubiquitin ligase FBXW7 in BTC samples and found a correlation with the metastasis status[97]. Interestingly, the authors demonstrated via *in vitro* and *in vivo* experiments in BTC cells, that silencing of FBXW7 resulted in both, an EMT and a CSC phenotype: the epithelial marker E-Cadherin was found to be down-regulated, whereas Vimentin was up-regulated. Regarding CSC markers, silencing of FBXW7 increased the expression of OCT4 and NANOG and enhanced tumor sphere formation capability of the tested BTC cells. Conversely, forced expression of FBXW7 reversed both, the EMT and the CSC phenotype[97]. Results from a study conducted by Matsushita and colleagues also suggests a role of the Hh pathway in EMT regulation in BTC: knockdown of the Hh pathway component SMO caused up-regulation of E-Cadherin and down-regulation of Vimentin, accompanied by decreased invasive potential of these cells[92]. Lastly, Kong *et al*[95] demonstrated an EMT phenotype (high Vimentin and low E-Cadherin protein expression) in side-population CSCs in GBC, further connecting the EMT process with the CSC phenotype.

CONCLUSION

Although several studies suggest the existence of BTC-specific CSC, additional independent studies should verify these results in functional BTC models, especially in xenograft experiments. Furthermore, due to the heterogenic character of BTC, it should be taken into account that multiple CSC subpopulations with potentially different genetic background and surface marker profiles may exist. Involvement of classical stemness pathways and complexes such as Hh, Wnt, NOTCH and the PRCs as well as of diverse miR species in generation and maintenance of CSC in BTC is very likely. However, up to now, only few studies directly associated the ascertained role of these factors for BTC development with CSC characteristics (Table 2). Moreover, future studies should also concentrate on the role of the TME in the creation and maintenance of potential CSC niches in BTC. The CSC status is strongly dependent on the microenvironment, meaning that CSC traits may very well be not a static, but rather a dynamic phenomenon in which the TME and CSC niche play an absolutely pivotal role[127]. In this light, limitations of current *in vitro* and *in vivo* models should always be kept in mind, as (artificial) imitation of an environment as complex and as dynamic as the TME seems very challenging. Current clinical trials involving the CSC concept mainly include immunotherapies, use of CSCs as biomarkers, several CSC-targeted therapies (*e.g.,* metformin, Hh or Notch inhibitors with/without concomitant chemotherapy). However, currently no studies are registered at clinicaltrials.gov specifically enrolling patients with BTC. In addition, future studies should also concentrate on the investigation of the role crosstalk between stemness pathways regarding BTC CSC as well as on the role of (chronic) inflammation in the formation and maintenance of a CSC phenotype in BTC, especially due to the fact that in BTC cells positive for CD133 or OCT3/4, enhanced levels of

inflammation-related DNA damage was observed[63].

Taken together, CSCs are promising and attractive targets for pharmacological intervention. Therefore, regarding BTC, after identification and validation of putative CSC populations, screening and testing of anti-CSC-targeting compounds in BTC models will be of great importance in order to develop new therapeutic strategies and approaches.

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MINIREVIEWS

Potential role of nutraceutical compounds in inflammatory bowel disease

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Abstract

Conventional therapies for inflammatory bowel diseases (IBD) involve combinations of pharmacologic agents such as aminosalicylates, azathioprine, and corticosteroids. Recently, the therapeutic scenario has been heavily increased by the introduction of agents including monoclonal antibodies targeted to specific proinflammatory cytokines, to adhesion molecules, and the induction of anti-inflammatory cytokines and T-cell activation. However, the use of these drugs is accompanied by a certain number of side effects, with some of them being quite severe, rising concerns about the safety profile. Furthermore, the cost of these emerging therapeutic strategies is significant , considering the increasing incidence and the chronic trend of IBD. Nutraceuticals is a broad term used to describe any product derived from food sources claiming extra health benefits beyond the intrinsic nutritional value found in foods. The beneficial effects of nutraceutical compounds in human health have been emerging in the last decades. Although few clinical trials have been performed in IBD patients, nutraceuticals, such as herbal products or vitamins, are generally accepted as safer alternative/supplementation to conventional therapy. In vitro and IBD-animal models studies have shown their involvement in several biological processes, including antioxidant defenses, cell proliferation, gene expression, which could account for a role in the maintenance of the mucosal barrier integrity, the control of the inflammatory pathways and the modulation of the immune response. These data suggest a wide spectrum of positive effects exerted by nutraceuticals, with a high potential for a therapeutic use in humans. In the present review, the beneficial effects of the most investigated nutraceutical compounds in the setting of human IBD are discussed.

Key words: Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Nutraceuticals; Probiotics; Phytochemicals; Herbals; Functional foods

Core tip: Current therapies for inflammatory bowel diseases (IBD) include aminosalicylates, azathioprine,

corticosteroids and recently clinical management with biologic agents has been implemented. However, safety issue are emerging along with concern about the high cost of these new drugs. Nutraceuticals is a broad term used to describe any product derived from food sources, such as herbal products or vitamins, with extra health benefits beyond the basic nutritional value. Despite few clinical trials in IBD patients, nutraceuticals are generally accepted as safer alternative/supplementation to conventional therapy and the available data support their high potential for therapeutic use in human IBD.

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INTRODUCTION

Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are a group of idiopathic, chronic and relapsing inflammatory disorders of the gastrointestinal tract, whose incidence and prevalence has been increasing in the last decades[1]. Although the etiology of IBD is still unclear, it is assumed that many interacting components could affect IBD development, including genetic susceptibility, ethnicity, environmental factors, infectious diseases, and dietary habits[2]. At present, the acknowledged pathogenetic mechanisms are featured by immune dysregulation, altered intestinal microflora, oxidative stress, defects in the gastrointestinal mucosal barrier and increased permeability, whose interplay leads to the onset of a state of chronic mucosal inflammation[3]. IBD patients often require lifelong medication, being the main goal of therapy both to induce a clinical remission and then maintain it for a long period of time. Severity and location of the disease account for the choice of therapeutic strategies, but also the awareness of potential side effects. Indeed, the currently approved drugs, such as corticosteroids, immunosuppressants and anti-tumor necrosis factor (TNF)-α antibodies, have been related to the risk of opportunistic infections and malignancies[4]. Furthermore, the course of the disease is often poorly controlled in a significant number of patients and besides conventional therapy, there are now emerging other novel biologic agents[5]. A crucial issue is the cost of these drugs, which are much more expensive than conventional therapy and concerns have arisen about the cost-effective management of IBD[6]. Surgery is the last option in critical patients, but is often associated with short- and long-term complications[7]. A poor adherence to therapy worsens this scenario, suggesting efforts by clinicians in understanding patients' needs and barriers to achieve a successful management[8]. For all the above mentioned reasons, alternatives for a safer, cheaper, and efficacious approach in managing IBD patients are being sought.

Nutraceutical compounds, such as bioactive peptides, phytochemicals, and omega 3-polyunsaturated fatty acids, are currently under investigation for their helpful activities in IBD[9]. According to the acknowledged definition, nutraceuticals are foods or parts thereof that provides medical or health benefits, including the prevention and/or treatment of a disease. The term "nutraceutical" was coined in 1989 by Stephen DeFelice and combines two words - "nutrient" (a nourishing food component) and "pharmaceutical" (a medical drug)[10]. The definition of nutraceuticals and related products generally depends on the source. They can be classified on the basis of their natural sources, or according their pharmacological conditions, as well as chemical constitution of the products. This means that different way of classification are possible. Chauhan *et al*[11] proposed that nutraceuticals can be grouped into three broad categories: (1) substances with established nutritional functions, such as vitamins, minerals, amino acids and fatty acids, also defined nutrients; (2) herbs or botanical products as concentrates and extracts, often called herbals; and (3) reagents derived from other sources (*e.g.,* pyruvate, chondroitin sulphate, steroid hormone precursors) serving specific functions, such as sports nutrition, weight-loss supplements and meal replacements, also indicated as dietary supplements (Figure 1). In recent years, use of complementary and alternative medicine (CAM) has spread due to the perceived natural and health benefits and the most commonly used CAM in IBD patients appear to be vitamin supplements and herbal therapies[12]. Previous studies in active IBD and in experimental DSS-colitis have shown that dietary supplements, such as probiotics, fish oil, curcumin and aloe vera, can ameliorate intestinal inflammation[13]. Plant-derived natural compounds carry out their protective and therapeutic effect through different molecular pathways, including anti-inflammatory and immunoregulatory mechanisms, anti-oxidative properties, and modulation of intracellular signaling transduction pathways[14]. Curcumin and green tea supplementation have been reported to be effective in reducing both IBD symptomatology and inflammatory scores, but strong evidence is limited[15]. The aim of this minireview is to describe the more common nutraceutical compounds used in IBD and to comment on recent findings for their possible applications in humans. We did not chosen to discuss data deriving from in vitro or animal studies, and the agents heading each of the four paragraphs represent compounds worthy of note for their use in human clinical trials investigating IBD.

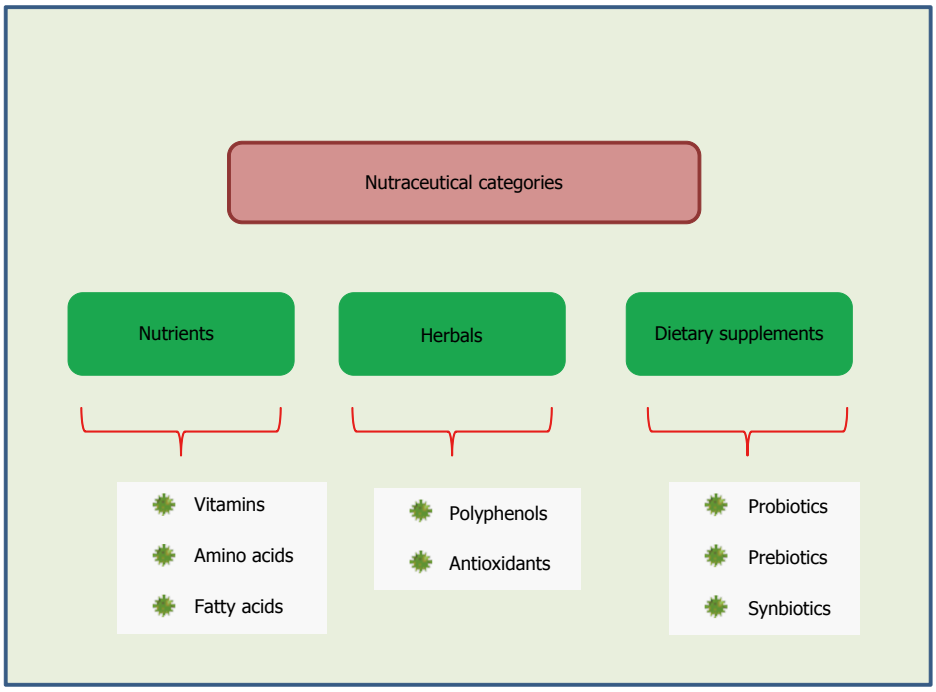

Figure 1 Macro-categories of Nutraceuticals and related most representative compounds.

PROBIOTICS AND PREBIOTICS

A growing body of evidence documents the use of probiotics and prebiotics in IBD treatment. Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host"[16]. The human gastrointestinal tract contains about 10^{14} bacteria, mostly concentrated in the large intestine and named intestinal microflora. It contributes to digestion of nutrients and waste products of metabolism and acts as an important barrier function against pathogens[17]. The triggering of chronic intestinal inflammation seems to depend somehow on the flora. In animal models, resident enteric bacteria are necessary for development of spontaneous colitis[18]. At the same time, a disruption of the fine balance between the host and its microbes is a hallmark of the inflammatory process in the gut[19]. Since this proven involvement of intestinal bacteria in IBD, various attempts have been made to modify the flora with probiotics. *Escherichia coli* (*E. coli*) *Nissle 1917*, *S. boulardii*, *L. casei*, *L. rhamnosus*, *Bifidobacterium*, represent some of the most studied microorganism in human IBD. In 1997, Malchow evaluated the maintaining remission rates among the probiotic and placebo groups using *E. coli Nissle 1917* in CD patients, but no statistically significant difference was found due to the small sample size[20]. A reduction in clinical relapse of CD to 6.25% *vs* 37.5% was obtained with *S. boulardii* supplementation over a 6 mo period but a larger subsequent study with the same agent did not confirm these benefits[21,22]. A third study with *S. boulardii* showed a reduction in the intestinal permeability in the probiotic group, however some concerns arose due to wide variability of intestinal permeability in CD patients and lack of correlation with CD activity index (CDAI) or endoscopic remission[23]. The largest probiotic trials in UC assessed the role of VSL#3, a multibacterial culture for oral use, consisting of the following species of bacteria: *B. breve*, *B. longum*, *B. infantis*, *L. acidophilus*, *L. plantarum*, *L. paracasei*, *L. bulgaricus*, and *S. thermophiles*[24]. An Indian multicenter trial showed a 42.9% of VSL#3 patients remission, compared with 15.7% of placebo patients[25], as well as an Italian 8 wk study which showed that VSL#3 was significantly superior to placebo in reducing the disease activity of mild-to-moderate UC. Moreover, VSL#3 improved rectal bleeding and seemed to lower entity of relapsing UC patients, although these parameters did not reach statistical significance due to the high placebo response rate and relatively short duration of the study[26]. *Bifidobacteria* was successful in maintaining UC remission in a long term trial, showing a 73% remission rate compared with 10% in the placebo group[27]. The probiotic drug *E. coli Nissle 1917* shows equivalent efficacy and safety in maintaining remission compared to the gold standard mesalazine in a large cohort of patients with UC. The ability in preventing relapse was confirmed by statistical analysis with both of the PP population and ITT analysis[28]. Similar findings were demonstrated by Rembacken *et al*[29] in 1999, who reported a 67% of relapse rate in the *E. coli Nissle 1917* group compared with 73% in the mesalazine group (P = 0.059). A lot of mechanisms have been proposed to explain the beneficial role of probiotics in IBD, focusing on their ability to colonize the colon and inhibit the growth of pathogenic

Table 1 Randomized controlled trials using probiotics, prebiotics and synbiotics in patients with inflammatory bowel diseaes

Ref.	Treatment	Duration	Subjects (*n*)	Findings
Malchow *et al*[20] 1997	*E. coli Nissle 1917*	12 mo	Active CD (28)	Trend toward reduced relapse rate
Guslandi[21] 2000	*S. boulardii*	6 mo	Active CD (32)	Significant reduction in relapse rate
Bourreille *et al*[22] 2013	*S. boulardii*	12 mo	Active CD (165)	No significant reduction in relapse rate
Garcia Vilela *et al*[23] 2008	*S. boulardii*	3 mo	Remission CD (34)	Reduction in intestinal permeability
Sood *et al*[25] 2009	VSL#3	12 wk	Active UC (147)	Significant achieved remission
Tursi *et al*[26] 2010	VSL#3	8 wk	Active UC (144)	Significant reduction in disease activity
Ishikawa *et al*[27] 2003	*Bifidobacteria*	12 mo	Remission UC (21)	Significant maintenance of remission
Kruis *et al*[28] 2004	*E. coli Nissle 1917*	12 mo	Remission UC (327)	Equivalence to mesalazine in remission
Rembacken *et al*[29] 1999	*E. coli Nissle 1917*	12 mo	Remission UC (166)	Equivalence to mesalazine in remission
Hafer *et al*[33] 2007	Lactulose	4 mo	Active CD and UC (31)	No clinical benefits
Benjamin *et al*[34] 2011	*Fructo-oligosaccharides*	4 wk	Active CD (103)	No clinical benefits
Steed *et al*[35] 2010	*B. longum plus inulin/oligofructose mix*	6 mo	Active CD (35)	Significant improvement in CDAI scores
Ishikawa *et al*[36] 2011	*B. breve plus galacto-oligosaccharide*	12 mo	Active UC (41)	Improvement of endoscopic score

E. coli: *Escherichia coli*.

species[30]. Moreover, probiotics are known to interact with epithelial and immune cells resident in the intestinal mucosa, reinforcing the barrier function and modulating the immune response[31]. Therapeutic modulation of the gut microbiota in IBD also contemplates the use of prebiotics, dietary substances that stimulate the growth and metabolism of protective commensal enteric bacteria, and synbiotics, which are products that contain both probiotics and prebiotics[32]. Hafer *et al*[33] showed no significant improvement in clinical activity index, endoscopic score or immunohistochemical parameters in CD and UC patients receiving lactulose compared with the control group. A similar failure was observed by the administration of fructo-oligosaccharides in 103 CD patients in a 4 wk trial[34]. A synbiotic consumption was effective in CD patients, ameliorating clinical outcomes and histological findings[35], and also in the UC setting synbiotic administration resulted in a promising outcome[36].

Table 1 summarizes the most relevant randomized controlled trials with probiotics, prebiotics and synbiotics, performed in patients with IBD in the last twenty years.

PHYTOCHEMICALS

Phytochemicals are a group of nutraceutical compounds derived from plants which hold healthy properties. Benefits from nutraceuticals rich in polyphenols and antioxidants derive from their properties to scavenge free radicals, induce anti-inflammatory responses, maintaining a homeostatic regulation of the gut microbiota, and activate the intestinal T regulatory cells[37]. Phenolic compounds represent the most widely distributed plant secondary metabolites and several studies have investigated their effects on intestinal inflammation, either as pure molecules or as plant extracts, in humans[38]. Curcumin is the principal natural curcuminoid (a class of phenols) found in the plant *Curcuma longa*, which is commonly used as a spice, food preservative and a coloring agent in foods. Its anti-inflammatory mechanism works mainly through the suppression of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)-related inflammatory pathway, with subsequent inhibition of TNF-α, IL-12 and IL-2, thus affecting the immune response modulation and representing a safe and promising agent for treatment of IBD[39]. Curcumin was reported to be effective in inducing remission in IBD patients both in a pilot study and in a multicenter randomized, placebo-controlled, double-blind trial, without producing adverse effects[40,41]. Moreover, curcumin showed a lower relapse rate in UC compared with placebo, supporting its efficacy also as a maintenance therapy[42]. Beside oral administration, curcumin was found to be effective also in enema formulation, as reported by Singla *et al*[43] in a randomized, double-blind, single-centre pilot study. Of note, the tolerability of oral supplementation with curcumin was assessed in a pediatric IBD population, and did not raise concerns regarding its safety[44]. *Aloe vera* gel, a plant extract known for medicinal purposes in several cultures for centuries, is one of the common herbal therapies used for IBD, despite a lack of large trials confirming its efficacy[45]. Langmead *et al*[46] demonstrated the induction of clinical response in UC patients after a four week treatment with orally *Aloe vera* gel administration, but no significant effects on endoscopic and histological outcomes were found. Flavonoids, which are widely distributed in fruits and vegetables, are included into the polyphenols category. Flavonoid sources as dietary supplement for therapeutic use have been proposed and their mode of action have been documented. Their use in IBD, thus limited in evidences, is intriguing due to the exhibited anti-inflammatory and immunomodulatory properties[47]. Anthocyanins are a class of dietary flavonoids widespread in fruits and flowers, where they are responsible for the blue, purple and red colours. Their anti-oxidant properties, added to a direct interference both with gene expression and receptor-

regulated signaling pathways of inflammation, have been recently investigated. During an open label pilot study, UC patients with mild to moderate disease were treated with an anthocyanin-rich bilberry preparation. After 6 wk treatment, endoscopic and histologic disease activity and fecal calprotectin levels were significantly reduced in the study participants[48]. Furthermore, colon biopsies of UC patients who responded to the 6 wk bilberry treatment revealed reduced amounts of the pro-inflammatory cytokines IFN-γ and TNF-α along with enhanced levels of the immunoregulatory cytokine IL-10[49]. Beside anthocyanins, other flavonoids exhibit nutritional values and a good safety profile of flavonoids from myrrh and chamomile has been found in a non-inferiority trial with IBD patients[50]. *Boswellia serrata* is a traditional herbal remedy with the recognized properties of intestinal epithelial barrier preservation and oxidative and inflammatory damage attenuation, and therefore in 2011 a large trial was carried out with this extract with the aim to maintain remission in CD patients[51]. Despite a good safety profile, no statistically significant difference was found compared with placebo regarding the primary endpoint. In a UC setting, an old study demonstrated an improvement of clinical parameters in the 30 patients treated with *Boswellia serrata* gum resin preparation compared with the controls who received sulfasalazine[52]. *Pistacia lentiscus*, known as Chios mastic gum, is an evergreen shrub widely distributed in the Mediterranean region. Oleogum resin from *Pistacia lentiscus* was found to act as an immunomodulatory agent on peripheral blood mononuclear cells by inhibiting TNF-α and stimulating macrophage migration inhibitory factor (MIF) activity. Indeed, its administration in CD patients resulted both in the reduction of TNF-α secretion and in the increase of MIF, with consequent inhibition of random migration and chemotaxis of monocytes/macrophages[53]. Clinical benefits of *Pistacia lentiscus* in CD was sustained by another study which showed a significant reduction in the CDAI compared with pretreatment values, following 4 wk of treatment[54]. The herbal extract HMPL-004 is derived from *Andrographis paniculata*, a herbal mixture used to treat inflammatory diseases, which has been reported to significantly reduce the transcriptional activity of NF-κB and decrease secretions of pro-inflammatory cytokines such as TNF-α and IL-6. Its benefits in active UC were addressed by two randomized, double-blind, 8 wk trials, showing a decrease in the total Mayo score of three points as well as a 30% reduction in rectal bleeding[55,56]. However, those promising greater rates of remission and clinical response compared with placebo or mesalazine did not reach statistical significance, thus suggesting a potential role for HMPL-004 in the management of IBD but also enforcing the need for additional and adequately powered studies. Figure 2 summarizes the interactions between some phytochemical agents and the inflammatory network.

DIETARY LIPIDS AND FAT-SOLUBLE VITAMINS

Dietary lipids are one of the most active nutritional substrates modulating the human immune response and, in particular, the gut mucosal immune system. The behavior of polyunsaturated fatty acids (PUFA) have been widely investigated during inflammatory processes and also in the IBD setting. The most interesting fatty acids are the n-6 PUFA arachidonic acid (AA), which is the precursor of inflammatory eicosanoids like prostaglandin E(2) and leukotriene B(4), and the n-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are abundant in fish oils. The modifying action of the lipid mediator profile is exerted by n-3 PUFAs mainly acting as a competitive substrate, which decreases the production of the eicosanoids from AA, but also reducing leucocyte chemotaxis and inflammatory cytokine production[57]. A rising incidence of IBD has been reported in countries in which the diseases were previously uncommon and this fact became more prominent after the "westernisation" of lifestyle, in particular with dietary changes including a higher intake of n-6 PUFAs and a reduced consumption of n-3 PUFAs[58]. Tjonneland *et al*[59] observed a correlation between higher intake of linoleic acid, a n-6 PUFA, and an increased risk of UC, suggesting a possible role for dietary linoleic acid in the etiology of the disease. On the other hand, oleic acid, which is the predominant ingredient of olive oil, was found to be inversely associated with UC development[60]. In agreement with this finding, a prospective large study showed an inverse association between greater long-term intake of long-chain n-3 PUFAs and risk of UC, confirming the protective effect of n-3 PUFA intake, while no specific fatty acids appeared to be associated with the risk of CD[61]. A Japanese study showed that a dietary intervention focused on lowering n-6/n-3 PUFA ratio was effective in maintaining disease remission in patients with IBD, possibly through the increasing of n-3 PUFA intake[62]. A 12 wk fish oil supplementation in patients with IBD resulted in n-3 PUFA incorporation into gut mucosal tissue and modification of inflammatory mediator profiles, showing how readily colonic lipids, prostaglandin and thromboxane synthesis can be altered by dietary changes[63]. Eleven patients with UC were studied in an 8-mo, double-blind, placebo-controlled, trial of dietary supplementation with fish oil, achieving a mean disease activity index that had declined in 56% of patients receiving fish oil and in 4% of patients on placebo. Moreover, fish oil ingestion revealed a well-tolerated profile in all patients and no alteration in routine blood exams appeared[64]. Despite these promising results, a large, randomized, multicenter trial did not show the utility of omega-3

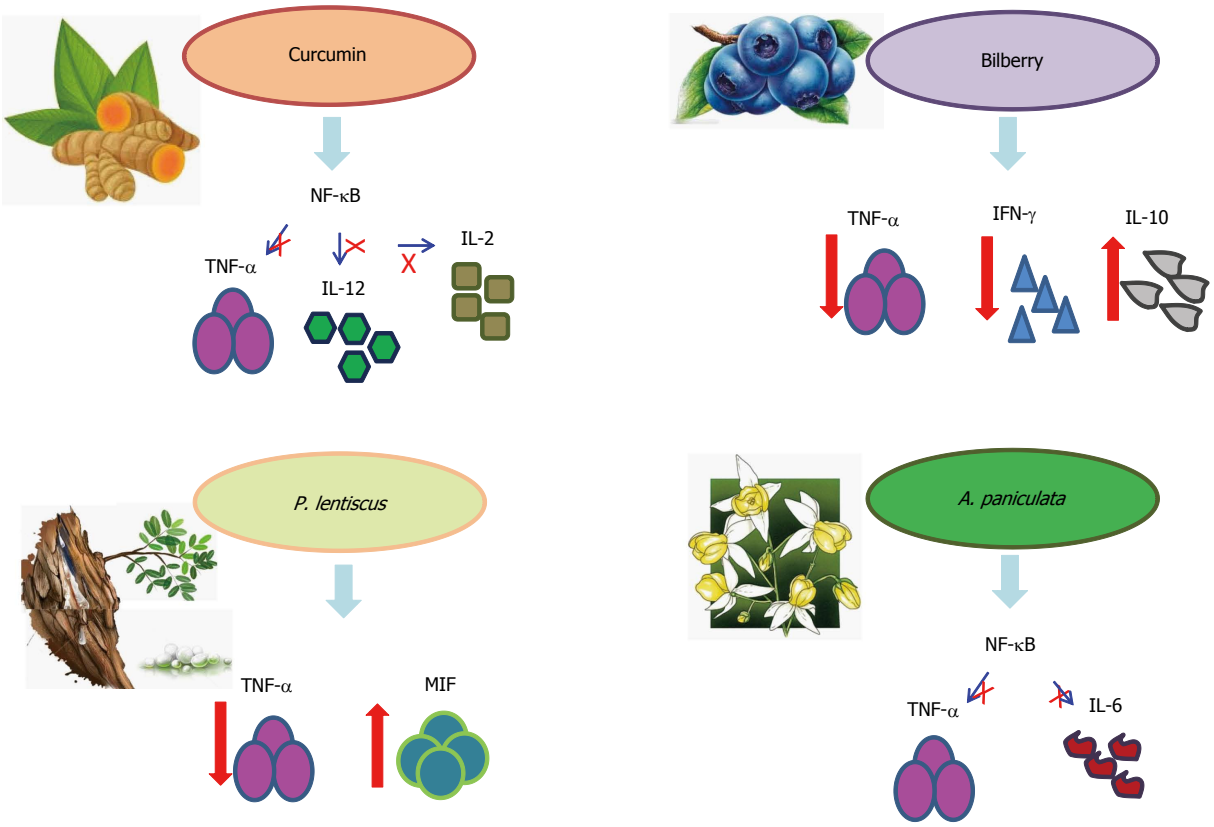

Figure 2 Phytochemicals and their interactions with inflammatory pathways. IFN-γ: Interferon-gamma; IL: Interleukin; MIF: Macrophage migration inhibitory factor; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; TNF-α: Tumor necrosis factor-alpha.

free fatty acids for the prevention of relapse in CD[65]. However , the above mentioned successful data need to be interpret with caution due to the small study size and poor study quality. Indeed, a recent review observed how the current data do not allow for a definitive conclusion regarding the efficacy of fish oil in IBD, there being not sufficient data to recommend the use of omega 3 fatty acids for treating those conditions[66].

An interesting role in the nutraceutical scenario used in the IBD setting has been proposed for the fat-soluble vitamins, such as A, D, E, and K. The deficiency of vitamin D, whose main source is endogenous production in the skin upon exposure to sunlight, was found to be significantly associated with IBD[67]. Evidence supports an immunological role of vitamin D in IBD, both promoting tissue barrier formation through the expression of cell adhesion proteins and stabilization of tight junctions between epithelial cells, and inhibiting the production of pro-inflammatory cytokines through the activation of vitamin D receptor[68]. Interventional human studies examining the effects of vitamin D supplementation on disease activity in CD showed lower relapse rates and improvement in CDAI[69,70]. Results from a recent randomized controlled trial support the benefits from vitamin D supplementation also in UC patients, since a decrease in ESR and CRP levels was found in the treatment group[71]. However, an optimal vitamin D supplementation protocol for patients with IBD remains undetermined, and large well-designed clinical trials and mechanistic studies are needed to determine if, and how, the promising data of literature can be translated into tangible clinical benefits[72]. Still less is available regarding the other fat-soluble vitamins. It has been suggested that vitamin K may be involved in the modulation of disease activity and maintenance of bone health in IBD patients. Nakajima *et al*[73] evaluated vitamin K levels of patients with IBD by measuring serum undecarboxylated osteocalcin and found a significant correlation with the clinical activity index of CD. The complexity of these interactions has been highlighted by Kuwabara *et al*[74], who found that a low plasma concentration of vitamin K was an independent risk factor for low bone mineral density in IBD patients, but these low levels were associated with the patients' fat intake, and not with the intake of vitamin K, thus suggesting a malabsorption rather than a poor dietary intake.

DIETARY PEPTIDES AND AMINO ACIDS

Dietary peptides and amino acids (AAs) have been shown to modulate intestinal immune functions and influence inflammatory responses, being involved in reducing inflammation, oxidative stress, and apoptosis in the gut[75]. Processes that lead to bioactive peptide release include *in vivo* enzymatic digestion in the

gastrointestinal tract both by human and microbiota enzymes, or *in vitro* food processing. Any protein source can produce bioactive peptides, milk being the best studied, but bioactive peptides from egg, fish, meat, algae or soy have also been reported[76]. Milk-derived products are already in clinical use for the treatment of IBD, such as casein-based enteral feeds which are used for the treatment of CD and whose efficacy might be due, in part, to the presence of the anti-inflammatory cytokine transforming growth factor-β[77]. Colostrum is a form of milk produced by the mammary glands of mammals just prior to calving. Bovine colostrum is a rich source of nutrients, antibodies, antimicrobial peptides (*e.g.*, lactoferrin, lactoperoxidase) and growth factors and its beneficial properties have been demonstrated during an initial study with UC patients treated by enema administration[78]. An amelioration in body composition, with a decrease in fat percentage, has been obtained in CD patients after whey and soy protein dietary supplementation[79]. Considering that a reduction of body fat contributes to the control of inflammation, these nutritional strategies may be useful as alternative or ancillary treatments in IBD. The current available information indicates a potential for food-derived peptides to counteract inflammation during the course of IBD, but further investigation is needed to clarify which peptides are responsible for the benefits and how they exert their impact . While experimental studies in animal models evaluating isolated AAs such as tryptophan, glutamine, and cysteine, offer promising data, their effects in IBD have been poorly documented in humans. The few available clinical studies show disappointing results with oral glutamine in CD and a controversial role for arginine[80].

CONCLUSION

New challenges lie ahead for clinical management of IBD, since an increasing emphasis on the safety of therapies for IBD is emerging from literature, hand in hand with the introduction of novel drugs. The available data concerning the administration of nutraceutical compounds in IBD patients support their beneficial effects accompanied by a good safety profile and invite clinicians to pay more attention to these opportunities. Indeed, the cost of treatment of IBD patients is continuously rising and nutraceutical approaches might represent a new effective and cheap treatment method. However, evidence-based information about their use is still lacking and more clinical studies to elucidate their role and optimal administration are needed.

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MINIREVIEWS

Unusual gastric tumors and tumor-like lesions: Radiological with pathological correlation and literature review

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Abstract

Although gastric tumors have overlapping radiologic appearances, some unusual tumors may present specific imaging features. Using multidetector computed tomography (MDCT), with water as a negative oral contrast agent and intravenous contrast medium, can provide critical information for the diagnosis of gastric diseases. In addition, MDCT can evaluate the involvement of the gastric wall and extragastric extent of the disease, as compared with gastroenteroscopy and double-contrast upper gastrointestinal study. Regarding lesion location and size, enhancing and growth patterns, presence of calcification or fat, and involvement of the gastric wall and adjacent structures, CT may provide useful information. In this review article, we review the relevant literature and discuss the CT features and the histopathologic findings of different types of gastric lesions. The lesions are divided into benign (glomus tumors, schwannomas, leiomyomas, and lipomas), malignant (gastrointestinal stromal tumors, mucinous carcinomas, lymphomas, and carcinoid tumors), and tumor-like lesions (ectopic pancreas and bezoar). Familiarity with imaging appearances and pathologic findings can help physicians make an accurate diagnosis.

Key words: Multidetector computed tomography; Stomach; Neoplasm; Adenocarcinoma; carcinoid; Lymphoma; Lipoma; Glomus tumor; Heterotopic pancreas; Schwannoma; Gastrointestinal submucosal tumor; Leiomyoma; Bezoar

Core tip: Diagnostic imaging of gastric tumors remains a practical challenge. However, in some cases of uncommon gastric tumors and tumor-like lesions, there are some specific radiographic features. Using the multidetector computed tomography, with water as a negative oral contrast agent and intravenous contrast medium, can provide critical information for the diagnosis of gastric diseases. Familiarity with the computed tomography features of these diseases facilitates accurate diagnosis and further management.

Lin YM, Chiu NC, Li AFY, Liu CA, Chou YH, Chiou YY. Unusual gastric tumors and tumor-like lesions: Radiological with pathological correlation and literature review. *World J Gastroenterol* 2017; 23(14): 2493-2504 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i14/2493.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i14.2493

INTRODUCTION

Diagnostic imaging of gastric tumors remains a practical challenge. The most common primary gastric tumors are gastric adenocarcinomas (> 90%)[1].

Esophagogastroduodenoscopy (EGD) is the main test used to find stomach cancer and perform a biopsy, and multidetector computed tomography (MDCT) is used to locate the lesion and determine the extent of the disease. Using MDCT to characterize the disease always leads to a long list of differential diagnoses because many overlapping characteristics have been shown to exist among various gastric tumors (Table 1).

However, in some cases of unusual tumors and tumor-like lesions, familiarity with the most relevant radiologic features with clinical information allows adequate characterization and diagnosis. Using the MDCT, with water as the negative oral contrast agent, can provide useful information regarding lesion location and size, enhancing and growth patterns, presence of calcification or fat, and involvement of the gastric wall and adjacent structures.

In this review article, we review the relevant literature and discuss the CT features and histopathologic findings of different types of gastric lesions. The lesions are divided into benign (glomus tumors, schwannomas, leiomyomas, and lipomas), malignant (gastrointestinal stromal tumors, mucinous carcinomas, lymphomas, and carcinoid tumors), and tumor-like lesions (ectopic pancreas and bezoar).

CT protocol of stomach studies

Dynamic MDCT with stomach distention is optimal for the study of stomach tumors. The stomach is distended with positive or negative oral contrast to avoid overlooking tumors[2]. The traditional positive oral contrast material may not mix uniformly with gastric contents, and mimics pseudotumors. In addition, high-attenuation contrast may mask subtle disease on contrast-enhanced images of the gastric wall[3]. Water and low-concentration barium sulfate are used as a negative oral contrast agent. We preferred to use water as a negative oral contrast agent for optimal assessment. Water is free and well tolerated. Before the CT scan, our patient was fasted for 4 h. After intravenous injection of hyoscine N-butylbromide (Buscopan, Boehringer International, Ingelheim, Germany) for slowing down gastrointestinal movement, the water (500 mL) was administered in a routine procedure to obtain gastric distention before the patient was laid down on the CT table.

The dynamic CT imaging for the gastric lesions was performed in three phases (non-enhanced, arterial, and portovenous). Non-enhanced imaging was obtained to provide a baseline for the degree of lesion enhancement as well as detecting the hemorrhage, calcification, and fat component of the lesion. The arterial phase was obtained 30 seconds after the injection of a dose of 2 mg/kg of nonionic contrast material at a rate of 2.5 mL/s, using an automated power injector. The portovenous phase was obtained 50 s after the contrast injection. The contrast-enhanced phases help in assessing the extent of involvement of the stomach, differentiating mucosal and submucosal tumors, determining the enhancing and growth patterns, and detecting distant metastasis and lymphadenopathy[4,5].

BENIGN TUMORS

Glomus tumors

Glomus tumors are modified smooth muscle cells that recapitulate perivascular glomus body cells. They are typically found in peripheral soft tissues, but can occur anywhere in the body[6]. In the gastrointestinal tract, glomus tumors are more common in the stomach as benign vascular submucosal tumors. The clinical symptoms are nonspecific. However, larger lesions are likely to be ulcerated, causing upper gastrointestinal bleeding. Surgical resection is typically curative.

On gastroenteroscopy, the glomus tumors appear as nonspecific submucosal lesions with a smooth surface[7]. Glomus tumors are typically small and solitary, and are commonly located in the gastric antrum. In pre-contrast CT, they are iso-dense to the stomach wall and manifest as solitary hypervascular lesions in the arterial phase, which persist in the portovenous phase in dynamic CT (Figure 1)[8]. Sometimes, they may exhibit a hemangioma-like "central fill-in" enhancement pattern in the delayed phase[9]. By contrast, adenocarcinoma is a relatively poor-enhancing mucosal tumor manifesting as a polypoid lesion with generalized mural thickening or focal mural thickening, with or without ulceration.

In histologic analysis, glomus tumors are composed

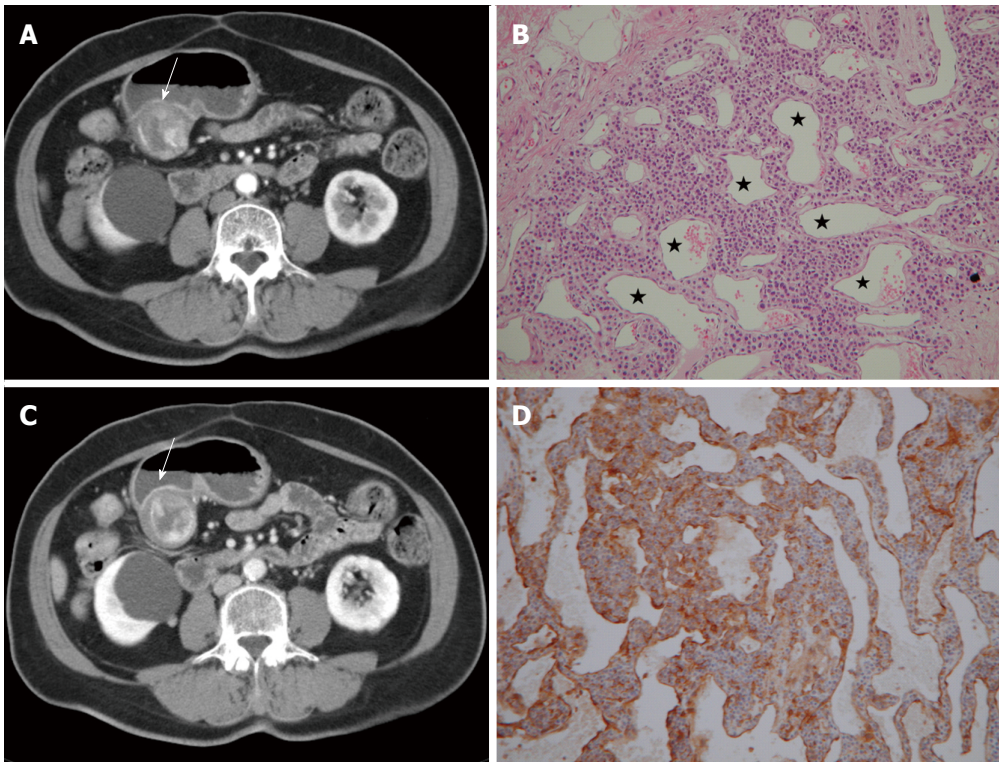

Figure 1 Glomus tumor. A 66-year-old woman presented with epigastric pain for 1 mo. A: Arterial phase showing a submucosal mass at the gastric antrum (arrow) with an exophytic growth pattern. Peripheral nodular enhancement is evident; B: Portovenous phase showing central fill-in enhancement compared with the arterial phase; C: High power photomicrography (original magnification, × 200, HE stain) showing many vessels (star) filled with red blood cells and lined within the tumor cells. The tumor cells were positive for smooth muscle actin (D).

Table 1 Characteristics of uncommon gastric tumors and tumor-like lesions

	CT features
Benign tumors	
Glomus Tumor	Small, solitary, and hypervascular tumor at gastric antrum
Schwannoma	Homogeneous attenuated gastric tumor
Leiomyoma	Small, endoluminal growth, hypoenhanced tumor at gastric cardia
Lipoma	Fat contained tumor
Malignant tumors	
Adenocarcinoma	Polypoid, or generalized mural thickening, or focal mural thickening with/without ulceration tumor The mucinous type has punctate or miliary calcification within the tumor
GIST	Exophytic hypervascular GI mass arising from submucosa with central ulceration, amorphous calcification
Lymphoma	Regional or diffuse wall thickening preserved perigastric fat plane and lymphadenopathy extending below the renal hila
Carcinoid	Type Ⅰ and Ⅱ, small, polypoid lesion, with marked enhancement. Type Ⅲ, larger, sporadic, solitary tumor with distant metastasis
Tumor-like lesion	
Ectopic pancreas	Small solitary lesion at greater curvature of distal antrum with enhancement similar to pancreas
Bezoar	Intraluminal gastric filling defect with mottled appearance

GIST: Gastrointestinal stromal tumor; CT: Computed tomography.

of numerous dilated, irregular shaped vessels, covered by numerous, monotonous round cells. The tumor cells are positive for smooth muscle actin (Figure 1)[6]. The prominent vascular structures are responsible for dense contrast enhancement in CT.

Schwannomas

Gastrointestinal schwannomas are considered to be different from conventional schwannomas because of unique different histologic features[10]. Conventional schwannomas are benign neurogenic tumors composed of over-proliferated Schwann cells in the soft tissue or central nervous system. In the gastrointestinal tract, such lesions often display prominent lymphoid cuffing (Figure 2). Gastrointestinal schwannomas are rare in the gastrointestinal tract, with the stomach being the most common site, followed by the colon and rectum. They are believed to arise from the gastrointestinal autonomic nervous system[11]. Gastrointestinal schwannomas account for 4% of all benign gastric

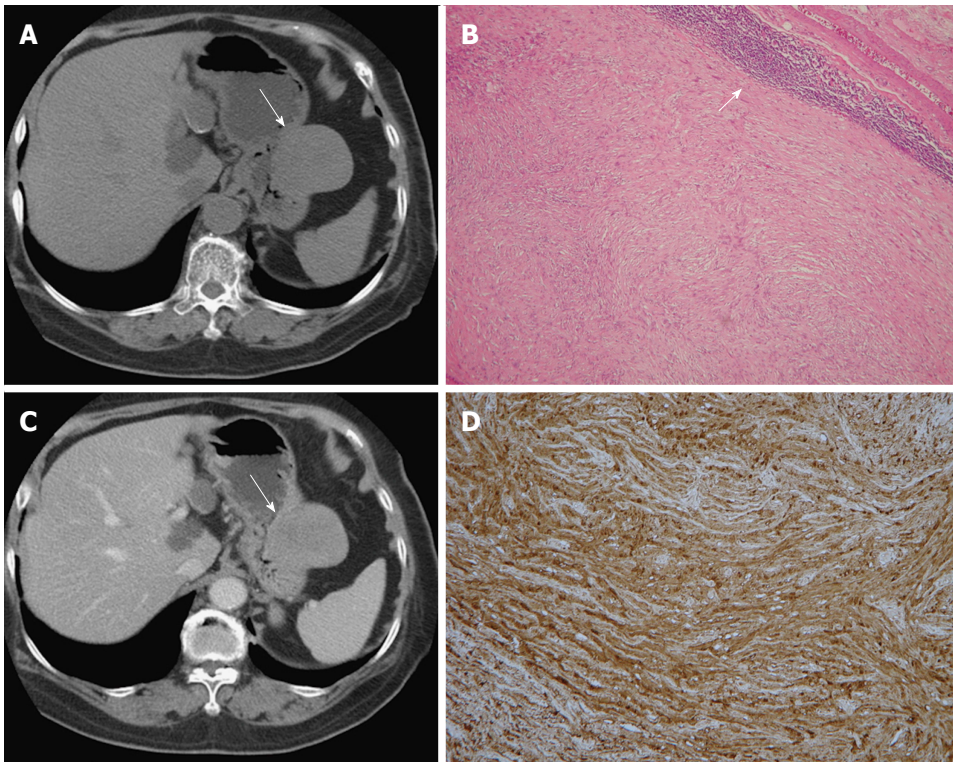

Figure 2 Schwannoma. A 75-year-old woman presented with coffee ground vomitus. A: Pre-contrast transverse computed tomography (CT) showing a homogeneous iso-density tumor in the greater curvature of the stomach (arrow); B: Post-contrast-enhanced CT showing homogeneously moderate enhancement with a mixed (endoluminal and exophytic) growth pattern; C: Low-power photomicrograph (original magnification, × 20; HE stain) showing that the tumor retains its circumscription with lymphoid aggregate cuffing (arrow); D: The vaguely bundled spindle tumor cells were positive for S-100.

neoplasms and the peak incidence occurs is in fourth and fifth decades of life[12]. They are typically asymptomatic, but can present with gastrointestinal bleeding or palpable mass.

Gastric schwannomas are submucosal lesions with endoluminal or exophytic growth patterns and their common CT appearance is homogeneous attenuation in pre-contrast and post-contrast images with moderate enhancement (Figure 2)[10]. Calcification, cystic change, hemorrhage, and necrosis are rarely seen in gastric schwannoma. By contrast, adenocarcinomas typically exhibit ulceration, necrosis, and involvement of the gastric mucosa.

In histologic analysis, gastrointestinal schwannomas consist of focally atypical spindle cells in a microtrabecular-microfascicular pattern with evidence of nerve sheath differentiation (S-100 protein-positive), peripheral lymphoid cuffing, and occasional germinal centers (Figure 2)[11,13].

Leiomyomas

True leiomyomas were not well distinguished from gastrointestinal stromal tumors (GISTs) until the development of immunohistochemical staining techniques. It is clinically important to distinguish GISTs from leiomyomas, because GISTs have a risk of progression and metastasis. True leiomyomas are benign neoplasms and never metastasize; surgery is not required unless obstruction or compression occur.

Gastrointestinal leiomyomas are the most frequent mesenchymal tumor of the esophagus[14]. They are relatively rare in the stomach, and are typically located in the gastric cardia. Leiomyomas typically manifest as homogeneous, low attenuation, poorly to moderately enhanced small masses in the gastric cardia (Figure 3). They typically exhibit an endoluminal growth pattern and are relatively small compared with GISTs[15].

Histologically, leiomyomas resemble to normal smooth muscle cells. They exhibit hypocellular spindle cells with eosinophilic cytoplasms, arranged in perpendicularly oriented fascicles. The tumor cells are positive for desmin and SMA, and negative for CD34 and CD117 (c-kit) (Figure 3)[16]. In contrast to true leiomyomas, GISTs show higher cellularity, and are positive for CD34 and CD117.

Lipomas

Gastrointestinal lipomas are benign submucosal tumors composed of adipose tissue covered with a fibrous capsule. They are solitary slow-growing tumors and can occur anywhere in the gastrointestinal tract. Approximately 90% to 95% of lipomas are located in the submucosa and the remaining 5% to 10% are subserosal[17]. They are rare in the stomach, and most

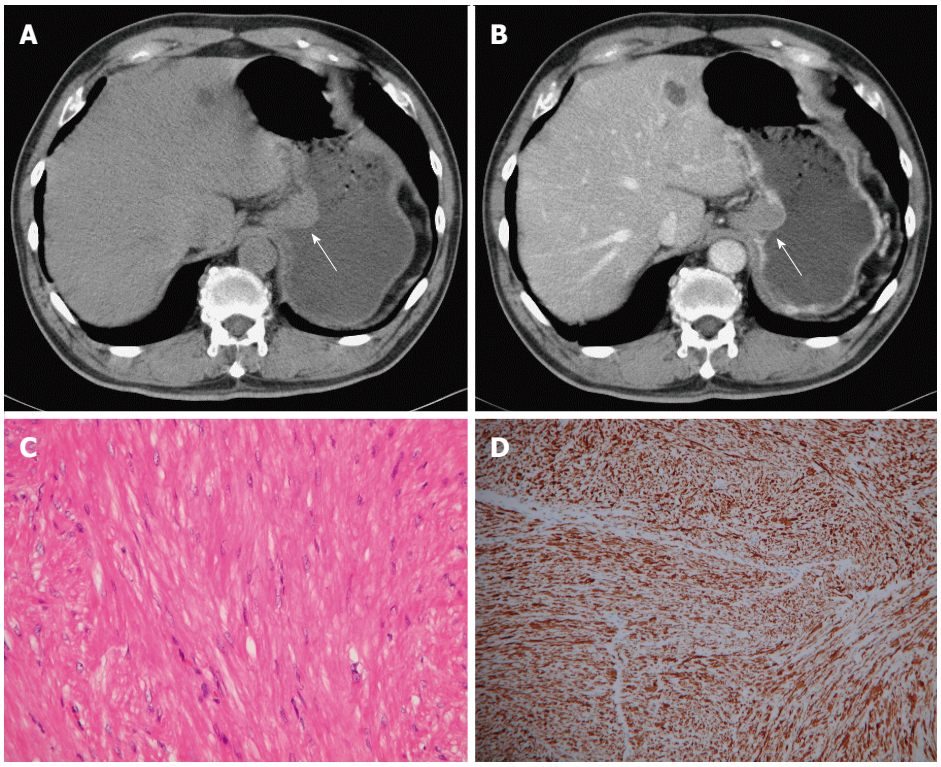

Figure 3 Leiomyoma. A 70-year-old man had no symptoms. A and B: Pre- and post-contrast-enhanced axial computed tomography scans showing the leiomyoma at the gastric cardia (arrow), with an intraluminal growth pattern and homogeneous, poor enhancement. Note the intact enhancing mucosa, indicating the submucosal lesion; C: High-power photomicrograph (original magnification, × 200; HE stain) showing paucicellular spindle cells with low or moderate cellularity, arranged in perpendicularly oriented fascicles; The tumor cells were positive for smooth muscle actin (D) and negative for CD34 and CD117 (not shown).

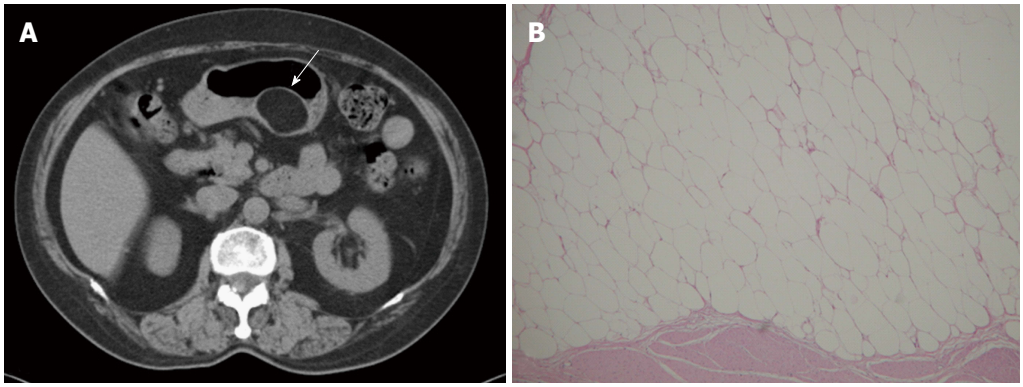

Figure 4 Lipoma. A 69-year-old man presented with abdominal fullness. A: Non-contrast-enhanced computed tomography (CT) showing a round, sharply marginated, uniform fatty mass (arrow) with negative CT numbers (-90 HU) in the greater curvature of the stomach; B: High-power photomicrograph (original magnification, × 200; HE stain) showing that the tumor consists of mature adipocytes.

are located in the gastric antrum with an endoluminal growth pattern[18]. Because lipomas are soft, they may prolapse through the pylorus into the duodenum without gastric outlet obstruction. If the tumor is large enough, it may cause intussusception. CT is the imaging modality of choice for diagnosing of lipoma. A definitive diagnosis is based on a well-circumscribed mass with uniform fat density (-70 to -120 HU) (Figure 4). Soft tissue attenuation may be present in the tumor because of inflammation and ulceration[19].

MALIGNANT TUMORS

Gastrointestinal stromal tumors

GISTs are the most common mesenchymal tumors of the gastrointestinal tract. GISTs can occur anywhere along the gastrointestinal tract, with approximately 60% to 70% occurring in the stomach, and 30% occurring in the small bowel. They arise from the intestinal cell of Cajal in muscularis propria of the gastrointestinal wall. Because they are mesenchymal

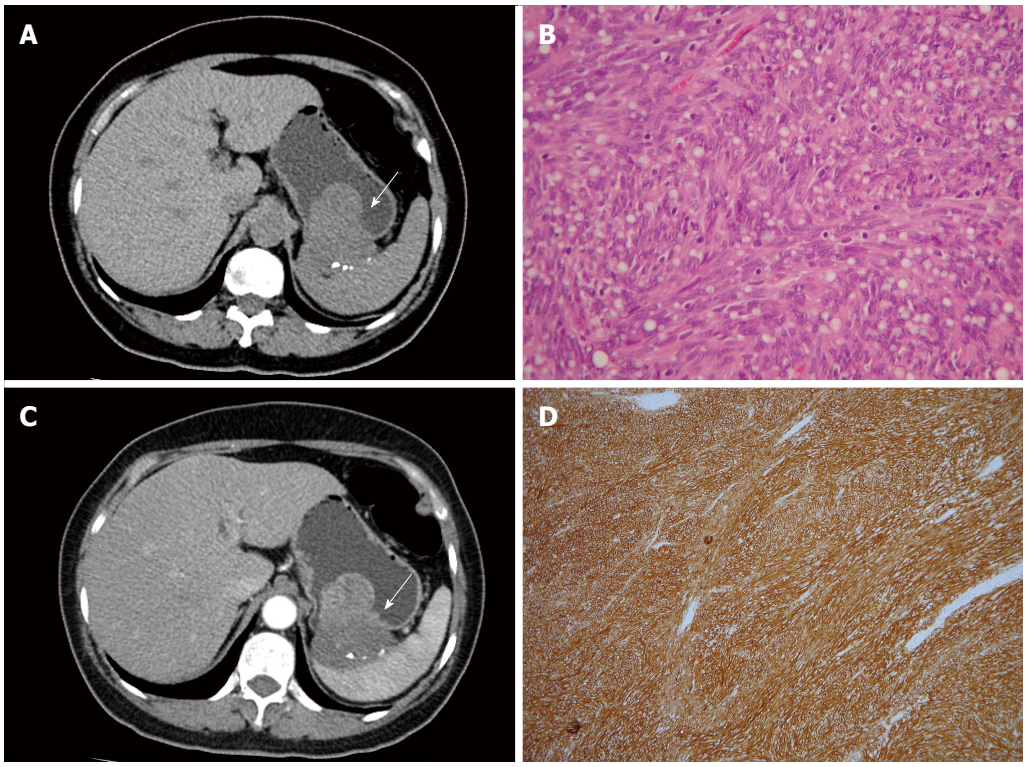

Figure 5 Gastrointestinal stromal tumor. A 58-year-old woman presented with melena and abdominal cramping pain for a year. A: Pre-contrast computed tomography (CT) scan showing amorphous calcifications in a gastric tumor with endoluminal and exophytic growth patterns (arrow); B: Post-contrast-enhanced CT scan showing the intact enhancing mucosa and central necrosis; C: High-power photomicrograph (original magnification, × 100; HE stain) showing spindle cells arranged in lobules; D: The tumor cells were positive for CD117.

tumors, they may exhibit exophytic, intraluminal or mixed growth pattern.

Primary GISTs are typically large masses with irregular lobulated margins, mucosal ulceration, central necrosis, hemorrhage, and heterogeneous enhancement (Figure 5). Occasionally, calcifications are observable and are amorphous. Sometimes it is difficult to identify the origin of a mass because of the large size and extraluminal growth pattern[20]. Nearly 50% of patients with GISTs exhibit metastasis, and the liver and peritoneum are the most involved organs[21]. GISTs smaller than 3 cm can be endoluminal and polypoid in appearance. They are typically well-defined, homogeneous, soft-tissue attenuation masses. Sometimes it is difficult to distinguish small GISTs from other benign intramural gastric tumors. Unlike adenocarcinoma, they are submucosal tumors with intact mucosa, prone to exhibiting mixed exophytic and endoluminal growth patterns and amorphous calcification.

Histologically, GISTs exhibit uniform spindle cells or epithelioid cells arranged in lobules. Malignant GISTs are larger, more highly cellular, and more mitotically active than benign GISTs. Most GISTs (90%) are characterized by expression of CD117 (c-kit), which is a tyrosine kinase receptor in the intestinal cells of Cajal (Figure 5). The immunoreactivity of CD117 (c-kit) distinguishes GISTs from true leiomyomas, leiomyosarcomas, schwannomas, and neurofibromas[22].

Mucinous adenocarcinomas

The gastric adenocarcinomas are classified into mucinous, papillary, tubular, signet-ring cell, and undifferentiated types. The prognosis of mucinous carcinoma is poorer than non-mucinous carcinoma. Mucinous carcinoma is characterized by prominent glandular formation and abundant extracellular mucin deposition[23]. Miliary and punctate calcifications are present in the mucin pool, which is a diagnostic clue for mucinous carcinoma (Figure 6)[24]. It is proposed that the alkaline mucin promotes calcium salt deposition[25], but the actual pathogenesis is not entirely clear.

Mucinous adenocarcinomas exhibit a diffuse thickened gastric wall with relatively low attenuation on pre-contrast-enhanced CT images and poor enhancement after contrast enhancement because of the accumulation of mucin (Figure 6)[26]. Calcifications within the tumor are rarely larger than 3 mm in diameter and are located within the thickened gastric wall. Calcifications in primary untreated gastric cancer are rare, but can sometimes be observed in GISTs and hemangiomas[27]. Calcification is amorphous in GISTs (Figure 5) and manifests as a cluster of phleboliths in hemangiomas, which differs from mucinous adenocarcinomas.

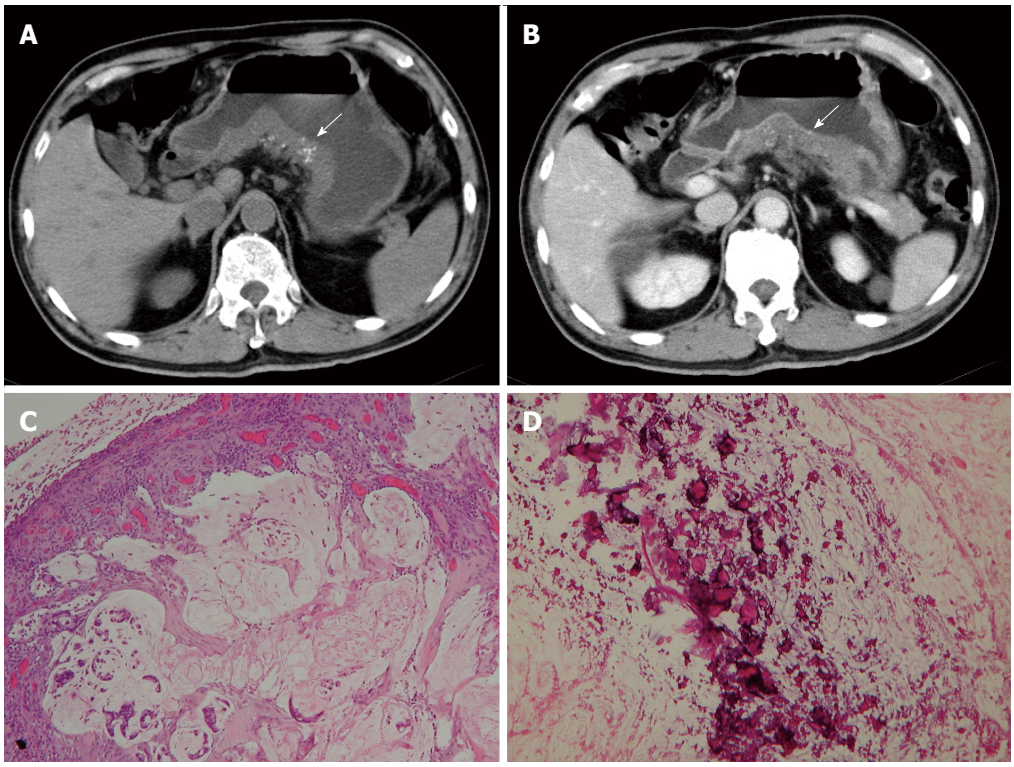

Figure 6 Mucinous adenocarcinoma. A 65-year-old man presented with vomiting and diarrhea for 2 mo. A and B: Pre- and post-contrast-enhanced computed tomography scans shoings a segmental thickening at the posterior wall of the gastric antrum, with poor enhancement and punctate calcification (arrow). Low-power photomicrograph (original magnification, × 20; HE stain) showing abundant extracellular mucin pools (C) with floating tumor cells and calcifications (D).

Lymphomas

Primary gastrointestinal lymphomas are the most frequently occurring extranodal lymphomas and are almost exclusively of non-Hodgkin type. The stomach constitutes 50% of all gastrointestinal tract lymphomas and 25% of extranodal lymphomas. Gastric lymphomas are predominantly non-Hodgkin lymphomas of B-cell origin[28,29]. It is believed that primary gastric lymphomas originate from low-grade mucosa-associated lymphoid tissue (MALT), and transform into intermediate or high-grade large cell lymphomas[30].

Gastric lymphomas typically show regional or diffuse gastric wall thickening on CT images (Figure 7). The enhancement is typically homogeneous, with preservation of the underlying gastric rugae, but low-attenuation areas of necrosis may be observed in some cases[31]. The stomach typically remains pliable and distensible, and transpyloric spread of the tumor may occur in 30% of cases[32]. In high-grade gastric lymphomas, involvement of adjacent organs is usually observed, with some perigastric lymph nodes. Low-grade MALT lymphomas frequently result in non-specific findings, such as mucosal nodularity, depressed lesions, and thickened folds. Compared to gastric adenocarcinomas, the perigastric fat plane is more likely to be preserved[33] and lymphadenopathy can typically be observed extending below the renal hilum (Figure 7)[31].

Carcinoids

Gastric carcinoids are well-differentiated endocrine neoplasms that originate from enterochromaffin-like cells in the gastric mucosa and are therefore epithelial in origin. Although the stomach is the least common site of gastrointestinal carcinoids, they are clinically important because of the associated carcinoid syndromes. Gastric carcinoids can be divided into three subtypes, each with a distinct pathophysiologic mechanism, resulting in different clinical outcomes and management[34,35].

Type 1 gastric carcinoids are the most common (75%-80% of gastric carcinoids), and are associated with hypergastrinemia and chronic atrophic gastritis. Patients with type 1 gastric carcinoid are typically asymptomatic; tumors are typically encountered during endoscopy for nonspecific symptoms. Type 2 gastric carcinoids are less common (5%-10% of gastric carcinoids) and are associated with Zollinger-Ellison syndrome, with multiple endocrine neoplasms. Approximately 30% of patients with multiple endocrine neoplasia type 1 have gastric carcinoid tumors[36]. In type 2 gastric carcinoids, elevated gastrin levels produce signs and symptoms of hypertrophic, hypersecretory gastritis. Type 1 and type 2 carcinoids are small, circumscribed, mucosal and/or submucosal

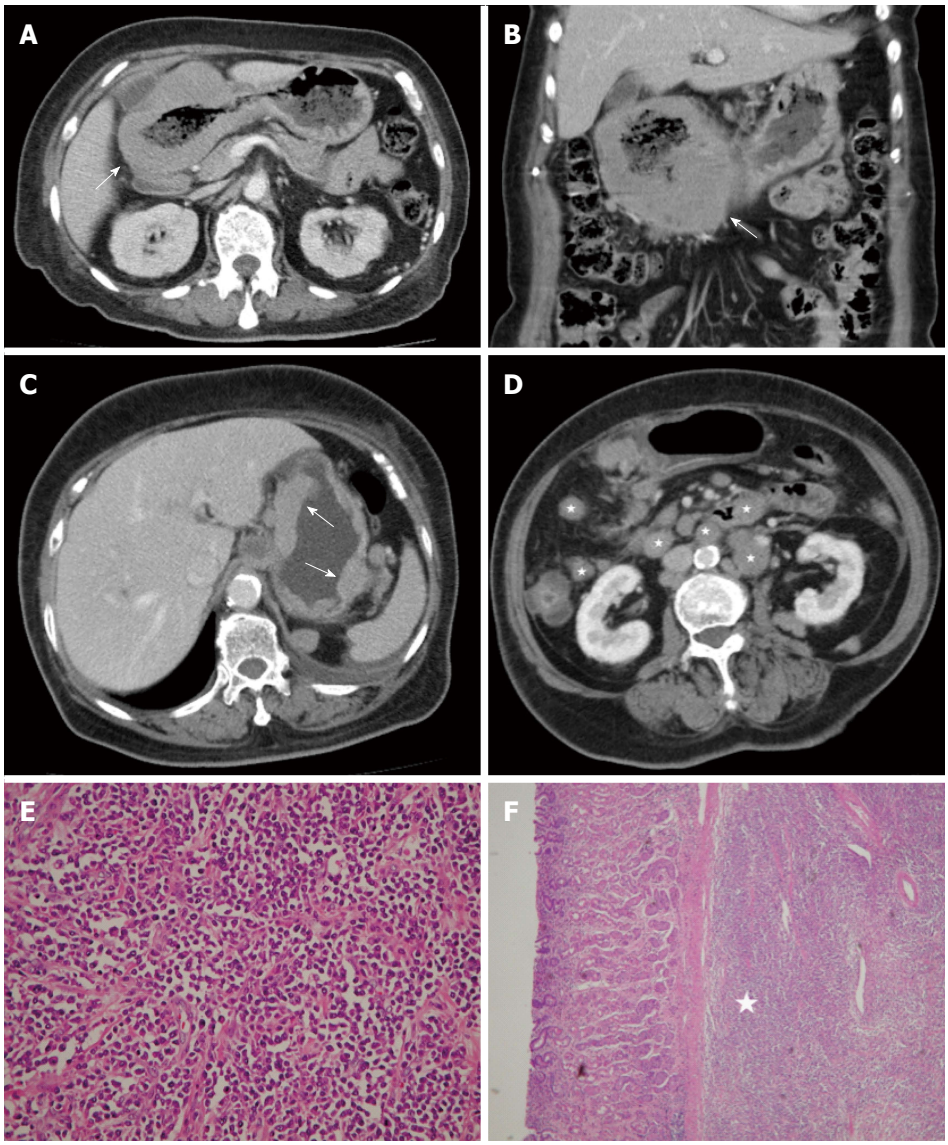

Figure 7 Diffuse large B-cell lymphoma. A 63-year-old woman presented with epigastralgia. A and B: Contrast-enhanced computed tomography (CT) scan showing diffuse, homogeneous gastric wall thickening with a smooth well-defined outer wall (arrow). An 83-year-old woman presented with tarry stool and constipation for a week; C and D: Post-contrast-enhanced CT revealing wall thickness (arrow) at the gastric body and several enlarged lymph nodes in the mesentery and para-aortic retroperitoneum (stars); E: Low-power photomicrograph (original magnification, × 20; HE stain) showing diffuse proliferation of large monomorphic neoplastic cells with abundant cytoplasms; F: The neoplastic cells occupy the full thickness of the submucosa (star).

polypoid tumors in the gastric body and fundus[37,38]. On CT, polypoid lesions appear iso-dense in the pre-contrast phase, with marked enhancement in the arterial phase (Figure 8)[39]. Type 3 gastric carcinoids, which are larger, sporadic, solitary tumors, are not associated with atrophic gastritis or hypergastrinemia; however, they may exhibit ulceration and distant metastases, and the prognosis is poor compared with type 1 and 2 carcinoids[40].

In histologic analysis, carcinoids are composed of small uniform cells arranged in trabecular or nest patterns. The nuclei are round or oval with finely stippled chromatin, infrequent mitoses, and minimal nuclear polymorphism (Figure 8). High-grade sporadic carcinoid tumors may resemble small cell carcinomas, with nuclear pleomorphism, hyperchromasia and higher mitotic activity. These tumors are immunoreactive to chromogranin A and synaptophysin, which are general neuroendocrine markers[38].

TUMOR-LIKE LESIONS

Ectopic pancreas

Ectopic pancreas is a condition whereby pancreatic tissues lack anatomic and vascular connections to the pancreas[41]. The pathogenesis is not clear, but some believe that during normal pancreatic development from evaginations, one or more evaginations may remain in the bowel wall. Others suggest that pancreatic

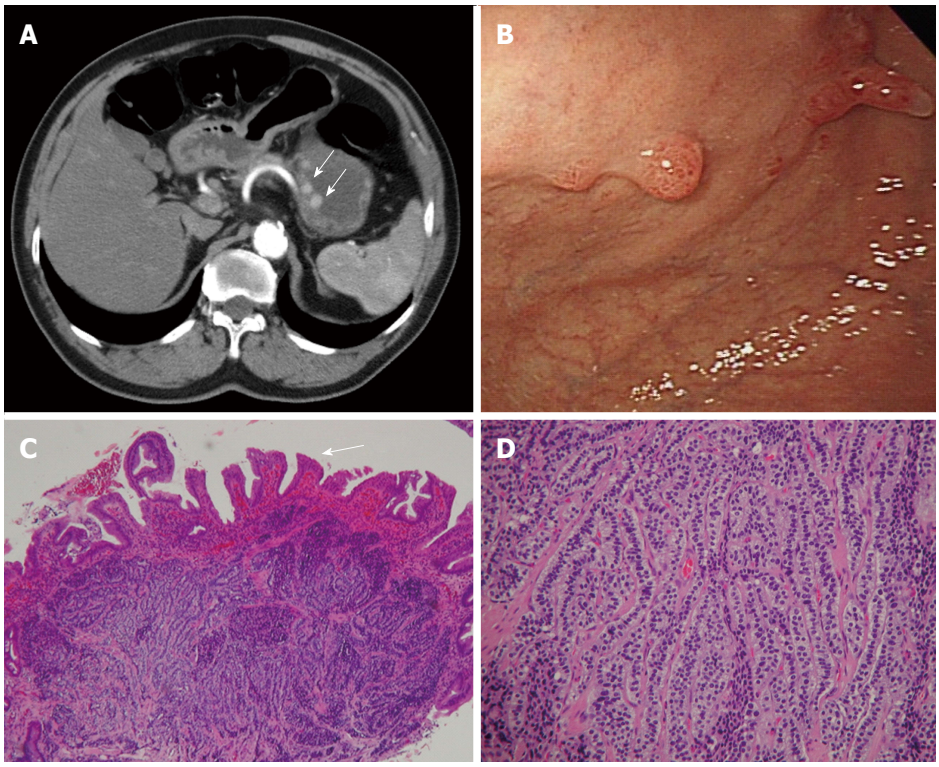

Figure 8 Carcinoid. A 66-year-old man presented with epigastralgia and elevated levels of serum gastrin. A: Contrast-enhanced transverse and coronal computed tomography (CT) scans showing multiple enhancing polypoid lesions (arrows) at the gastric body; B: Endoscopy showing multiple polypoid lesions; C: Low-power photomicrograph (original magnification, × 10; HE stain) showing atrophic gastritis (atrophy in glandular structures, arrow); D: High-power photomicrograph (original magnification, × 100; HE stain) showing uniform cells bearing round nuclei and growing in a festoon or ribbon-like arrangement in the submucosa.

metaplasia of the gastric submucosa may occur during the embryogenesis of endodermal tissues[42]. These lesions typically have a peak incidence in fourth to sixth decades of life, most commonly among men. They are typically discovered incidentally during surgery or autopsy, with an incidence during laparotomy of 0.2%[43]. Although most patients with ectopic pancreas are asymptomatic, some may present with nonspecific abdominal pain, bleeding, and mechanical obstruction[44-46]. Lesions are typically located in the stomach, duodenum, or jejunum. Because they are typically small, slow-growing, and asymptomatic, they might be overlooked in daily practice.

Ectopic pancreas typically manifests as an ill-defined, submucosal mass with endoluminal growth in the stomach, generally located in the greater curvature of the distal antrum[47]. On CT, the enhancement is typically similar to that of the normal pancreas. It has been reported that the enhancement degree depends heavily on the histopathologic composition of the ectopic pancreas[48]. There are three subtypes of ectopic pancreas. The acini-dominant type is more homogeneous and exhibits stronger enhancement than the normal pancreas (Figure 9), the duct-dominant type exhibits lower enhancement than the normal pancreas, and the mixed type exhibits variable CT attenuation values compared with the normal pancreas. Histologically, ectopic pancreas is not a diagnostic problem when pancreatic acini, ducts, islets of Langerhans, and intervening connective tissue are present.

Bezoar

Bezoars, mimicking gastric neoplasms, consist of ingested foreign materials that accumulate within the gastrointestinal tract. They include trichobezoars, which are composed of hair; phytobezoars, which are composed of fruit or vegetable matter; lactobezoars, which are undigested milk concretions; and pharmacobezoars, which are composed of medications[49]. They are typically confined to the stomach but can extend through the pylorus into the jejunum, ileum and even up to the colon (known as Rapunzel syndrome).

Most bezoar cases are diagnosed using plain films or a barium meal, but CT may be requested for patients who present with abdominal masses. A bezoar in the stomach presents as a mobile intraluminal gastric filling defect, with a mottled appearance caused by air bubbles retained in interstices of the mass. (Figure 10)[50]. Clinical history is crucial for accurate diagnosis.

CONCLUSION

It is often difficult to determine the etiology of gastric lesions based on the basis of clinical findings. Using

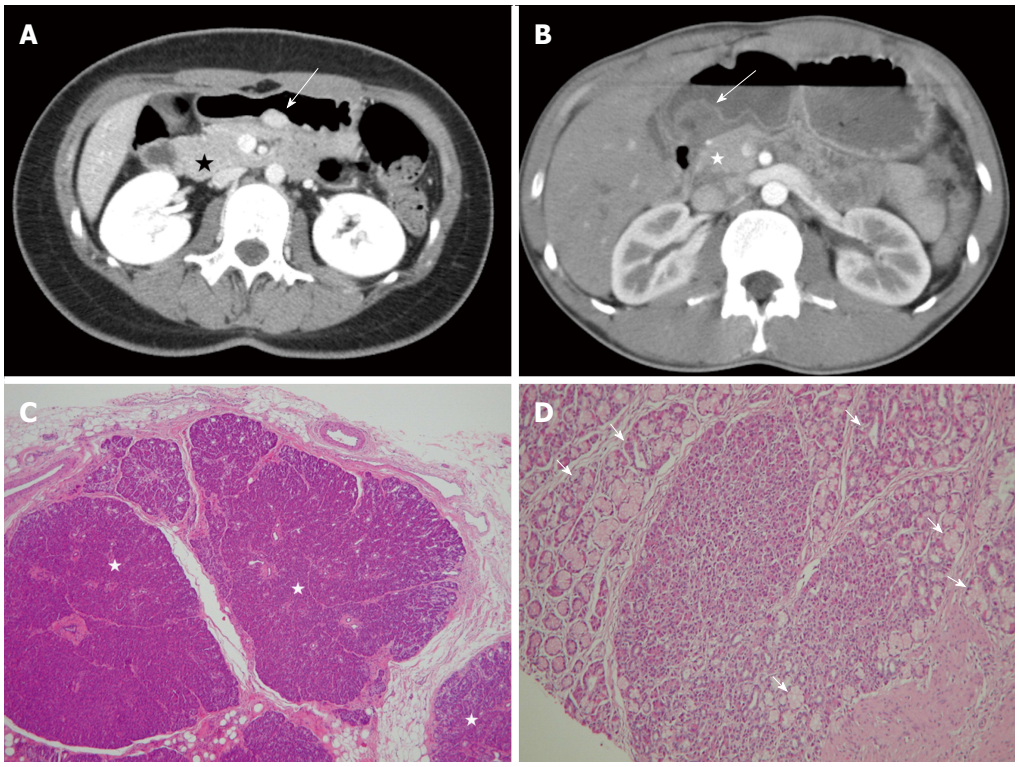

Figure 9 Ectopic pancreas. A 26-year-old woman presented with postprandial epigastric pain for 2 years. A: Transverse computed tomography (CT) scan showing a small round submucosal lesion with well-defined margins in the wall of the antrum (arrow). Note the contrast material enhancement is higher than that of the normal pancreas (star); B: Low-power photomicrograph (original magnification, × 20; HE stain) showing that pancreas tissue (star) is predominant in the acinar tissue. A 20-year-old man presented with intermittent epigastralgia for 2 mo; C: Transverse CT scan showing a submucosal round mass (arrow) with necrosis at the gastric antrum. Note the poorly enhancing nodular mass, as compared with the markedly enhancing adjacent normal pancreas (star); D: Low-power photomicrograph (original magnification, × 200; HE stain) showing ectopic pancreatic tissue, composed primarily of pancreatic ducts (arrow) in the gastric mucosal layer.

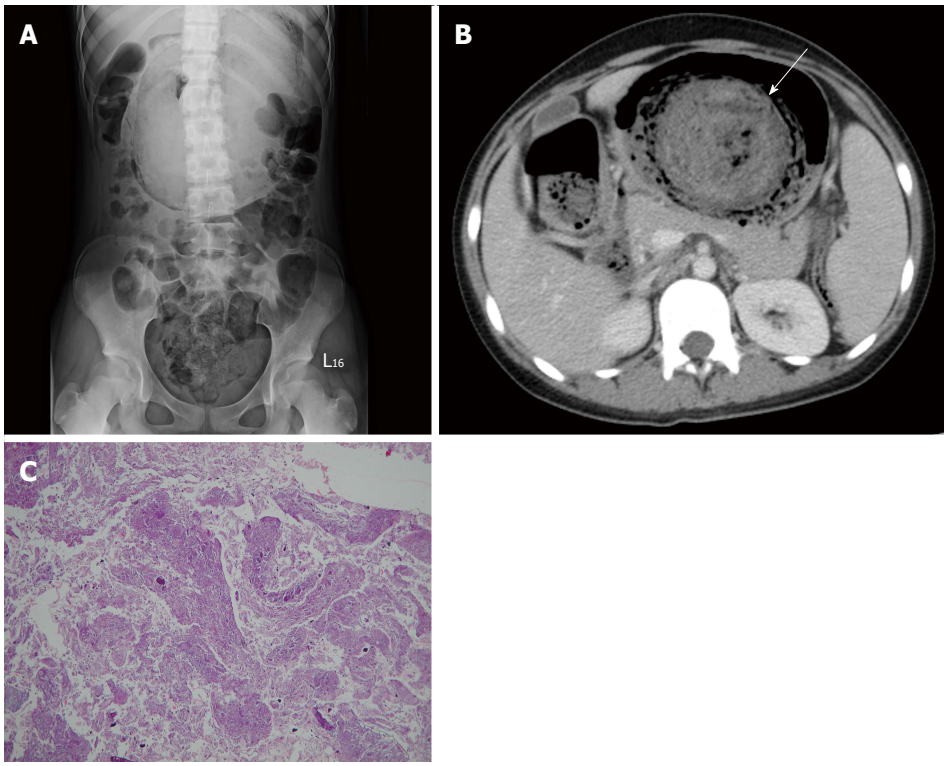

Figure 10 Trichobezoar. A 13-year-old girl presented with intermittent fever. She exhibited obsessive and compulsive hair pulling. A: Plain film revealing a bezoar outlined by air in the stomach; B: Transverse computed tomography image showing an inhomogeneous mass with a mottled gas pattern in the distended stomach (arrow); C: Low-power photomicrograph (original magnification, × 10; HE stain) showing hair tissue with inflammatory exudate.

MDCT with water as a negative oral contrast can provide useful information for the diagnosis of unusual gastric tumors or tumor-like lesions. Familiarity with imaging appearances and pathologic findings can help physicians make an accurate diagnosis.

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MINIREVIEWS

New progress in roles of nitric oxide during hepatic ischemia reperfusion injury

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Abstract

Hepatic ischemia reperfusion injury (HIRI) is a clinical condition which may lead to cellular injury and organ dysfunction. The role of nitric oxide (NO) in HIRI is complicated and inconclusive. NO produced by endothelial nitric oxide synthase (eNOS) activation plays a protective role during early HIRI. But eNOS overexpression and the resulting excessive NO bioavailability can aggravate liver injury. NO induced by inducible nitric oxide synthase (iNOS) may have either a protective or a deleterious effect during the early phase of HIRI, but it may protect the liver during late HIRI. Here, we reviewed the latest findings on the role of NO during HIRI: (1) NO exerts a protective effect against HIRI by increasing NO bioavailability, downregulating p53 gene expression, decreasing inflammatory chemokines, reducing ROS *via* inhibiting the mitochondrial respiratory chain, activating sGC-GTP-cGMP signal pathway to reduce liver cell apoptosis, and regulating hepatic immune functions; (2) eNOS protects against HIRI by increasing NO levels, several eNOS/NO signal pathways (such as Akt-eNOS/NO, AMPK-eNOS/NO and HIF-1α-eNOS/NO) participating in the anti-HIRI process, and inhibiting over-expression of eNOS also protects against HIRI; and (3) the inhibition of iNOS prevents HIRI. Thus, the adverse effects of NO should be avoided, but its positive effect in the clinical treatment of diseases associated with HIRI should be recognized.

Key words: Liver; Hepatic ischemia reperfusion injury; Nitric oxide; Nitric oxide synthase

Core tip: The latest findings on the role of nitric oxide (NO) during hepatic ischemia reperfusion injury (HIRI) include: NO exerts a protective effect against HIRI by increasing NO bioavailability, downregulating p53 gene expression, decreasing inflammatory chemokines, reducing ROS by inhibiting the mitochondrial respiratory chain, activating sGC-GTP-cGMP signal pathway to reduce liver cell apoptosis, and regulating hepatic immune functions; eNOS protects against HIRI by increasing NO levels, several eNOS/NO signal pathways (such as Akt-eNOS/NO, AMPK-eNOS/NO and HIF-1α-eNOS/NO) participating in the anti-HIRI process; inhibiting over-expression of eNOS also protects against HIRI; and finally, the inhibition of iNOS prevented HIRI.

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INTRODUCTION

Hepatic ischemia reperfusion injury (HIRI) is a clinical condition which may lead to cellular injury and organ dysfunction, mediated mainly through the production of reactive oxygen species and inflammatory cytokines[1]. Deterioration of hepatic homeostasis, as observed in IR, cold preservation and transplantation, septic organ failure, and hepatic resection-induced hyperperfusion, are associated with high rates of morbidity and mortality. It is well known that HIRI involves several mechanisms, which include pH imbalance, Ca^{2+} overload, mitochondrial damage induced by oxygen free radicals, endothelin (ET)/nitric oxide (NO) ratio imbalance, liver microcirculation dysfunction, activation of Kupffer cells and neutrophils, and the impact of various cytokines. During IR, there are interactions among liver cells, Kupffer cells, neutrophils, hepatic sinusoidal endothelial cells, and fat-storing cells. Platelets and alexin are also involved[2]. These activated cells release a large quantity of proinflammatory cytokine and lipid inflammatory factor, which can lead to inflammatory reaction and cell apoptosis.

NO is an unstable carbon-centered radical with a short half-life. There are two sources in organisms: one is non-enzymstigenese derived from the degradation or transformation of inorganic nitrogen chemicals in the body and food; and the other one is enzymstigenese, in which NO is produced in a redox reaction between L-arginine and oxygen molecules by NO synthase (NOS) catalyzation. There are three types of NOS: endothelial nitric oxide synthase (eNOS), neuronal nitric oxide synthase (nNOS), and inducible nitric oxide synthase (iNOS). Endothelial nitric oxide synthase exists mainly in vascular endothelial cells while iNOS exists mainly in the cytoplasm of some inflammatory cells, such as white blood cells associated with diseases characterized by inflammation, tumors, and degeneration. NOS not only appears in soluble cytoplasm but also in some subcellular organelles. NO is produced mainly by eNOS catalysis, and also by the upregulation of iNOS expressions during acute hepatic ischemia[3]. It has been reported that iNOS is induced to produce large amounts of NO by lipopolysaccharide, interleukin-1 (IL-1), and tumor necrosis factor (TNF), which play a role in the pathophysiological process of some diseases and in many inflammation and immune reactions[4]. iNOS-derived NO may have either a protective or a deleterious effect during the early phase of IR injury, but it plays a protective role in the late phase of HIRI[5] (Table 1).

PROTECTIVE EFFECT OF NO DURING HIRI

NO was proved to reduce HIRI through various mechanisms[6,7], such as inhibiting liver cell apoptosis, slowing the infiltration of macrophages, eliminating superoxide anion produced by neutrophils, protecting the liver sinus structure and maintaining liver microcirculation blood flow, accelerating the liver tissue oxygenation, stabilizing ATP levels, decreasing oxidative stress injury, preventing the reduction of glutathione and the increase of endothelin side effects, and inhibiting platelet aggregation.

Increase of NO bioavailability involved in its protective effect in HIRI

In addition to NO donors, an increase in NO bioavailability can also protect the liver from HIRI. Human serum albumin (HSA) is a non-glycosylated protein, by which a series of recombinants, and mannosylated-HSA mutants (Man-rHSAs) are prepared; their triple mutant (TM-rHSA) can be selectively delivered to the liver *via* a mannose receptor on non-parenchymal liver cells, which can effectively deliver NO to the liver and have a significant inhibitory effect against HIRI[8].

NO downregulates the expression of the p53 gene and decreases inflammatory chemokines

During IR, NO donors may decrease p53 gene expression and the levels of IL-1 and TNF-α as well as inhibit cell apoptosis to protect the heart, liver, lungs, and kidneys from IR injury. Elevated NO levels can inhibit p53 gene expression and decrease the production of proinflammatory cytokines and chemokines, such as intercellular adhesion molecule (ICAM), TNF-α, IL-1, MIP-1, and MIP-2. In particular, lower levels of ICAM,

Table 1 Roles of nitric oxide, endothelial nitric oxide synthase and inducible nitric oxide synthase in pharmacological protection against hepatic ischemia reperfusion injury

Pretreatment	NO/iNOS/eNOS levels	Animals	Experimental cells	Mechanism	Liver cell necrosis and liver damage	Ref.
L-arginine and HIRI	NO↑	Male Sprague-Dawley rats	Hepatocytes	O2-↓, NO2-/NO3- concentration↑	↓	[6]
L-NAME and HIRI	NO↓	Male Sprague-Dawley rats	Liver cells	NO2-/NO3- concentration↓	↑	[7]
Human serum albumin↑	NO↑	Rats underwent HIRI	Liver parenchyma	Man-rHSAs↑	↓	[8]
SNAP and HIRI	NO↑	-	Vein endothelial cells	ICAM, TNF-α, NF-κB, p38, ERK, JNK, p53, caspase-3↓	↓	[9,10]
Nitrite and hypoxia/reoxygenation	NO↑	Trachemys Acripta elegans	Various cell types	Cytochrome oxidase, oxygen radical↓	↓	[13]
L-arginine and HIRI	NO↑	-	Liver cells	sGC, cGMP, PKG, PI3K, V-ATPase ↑, intracellular Na+, H+↓	↓	[5]
L-arginine and HIRI	NO↑	Rats	Liver cells	TNF-α, IL-1β↓	↓	[14,15]
HIRI	eNOS,NO↑	-	Bovine aortic endothelial cells and COS-7cells	Intracellular Na+, H+, PKC, Ca2+↑	↓	[16,17]
rHuEPO and HIRI	NO↑	Adult male Sprague-Dawley rats	Liver cells	PI3K/Akt/eNOS pathway	↓	[18]
Institut Georges Lopez-1 and HIRI	eNOS↑	Adult male SD rats	Liver cells	Akt,AMPK↑	↓	[19]
Adiponectin and HIRI	eNOS↑	Adult male Wistar rats	Hepatocytes	AMPK/eNOS pathway	↓	[20]
Heparin cofactor Ⅱ and ischemia	eNOS↑	Male heterozygote HC Ⅱ-deficient mice and male littermate WT mice	Vascular endothelial cell	AMPK/eNOS signaling pathway	↓	[21]
Trimetazidine, IGL-1, and HIRI	eNOS,NO↑	Isolated perfused rats liver model	Steatotic and non-steatotic livers cells	HIF-1α, heme-oxygenase-1↑	↓	[22]
Knockdown of AK139328 and HIRI	eNOS↑	Mice	Liver cells	p-eNOS, p-Akt, PGSK-3 ↑,macrophage infiltration, NF-κB↓	↓	[23]
Ad-eNOS and HIRI	eNOS↑	Male inbred C57BL/6 lean mice	Liver cells	ATP↓, bax↑	↑	[24]
Riboflavin and HIRI	eNOS,iNOS,NO↓	Mice	Liver	GSH↑	↓	[25]
Rosmarinic acid and HIRI	eNOS,iNOS,NO↓	Rats	Liver	eNOS excessive expression↓ NF-κB activity, TNF-α and IL-1β gene expression↓	↓	[26]
Alpha lipoic acid and HIRI	iNOS,NO↓	Male Wistar strain rats	Hepatocytes	iNOS mRNA stability↓	↓	[31]

-: No data; Man-rHSAs: Mannosylated-HSA mutants; HIRI: Hepatic ischemia reperfusion injury; IGL-1: Institut georges lopez-1; SNAP: S-nitroso-N-acetylpenicillamine; ICAM: Intercellular adhesion molecule; Akt: Protein kinase B; AMPK: Adenosine monophosphate-activated protein kinase; TNF-α: Tumor necrosis factor-α; NF-κB: Nuclear factor-κ-gene binding; ERK: Extracellular regulated protein kinase; JNK: c-Jun N-terminal kinase; PI3K: Phosphoinositide 3-kinase; V-ATPase: Vacuolar H^+-ATPase; rHuEPO: Recombinant human erythropoietin; HIF-1α: Hypoxia inducible factor 1α; PGSK-3: Phosphorylated glycogen synthase kinase 3; eNOS: Endothelial nitric oxide synthase; iNOS: Inducible nitric oxide synthase.

MIP-1, and MIP-2 are accompanied by less neutrophil infiltration[9,10]. The application of ICAM-1 monoclonal antibody 1A29F is likely to provide a more effective treatment for primary grafted liver dysfunction[11]. NO can also reduce the level of TNF-α to inhibit NF-κB. Decreased NF-κB can inhibit MAPKs, including p38, ERK, and JNK. A reduced p38 level can lead to the inhibition of caspase-3 and gene p53 expression. Thus, the downregulation of p53, ERK and JNK results in reduction of cell inflammation[9].

NO reduces ROS by inhibiting the mitochondrial respiratory chain

Using transgenic knockout rats, Datta studied the molecular mechanism of HIRI. It was found that the initial liver injury is initiated by reactive oxygen species, which cause direct cellular injury and also activate a cascade of molecular mediators, leading to microvascular changes, increased apoptosis, and acute inflammatory changes with increased hepatocyte necrosis. However, during the period of reperfusion, some adaptive changes occur in order to reduce HIRI[12]. Exogenously administered NO donors can inhibit the oxidation of mitochondrial cytochrome and reduce ROS production. Excessive ROS is generated in liver cells after its hypoxia/reoxygenation, which causes protein oxidation and lipid peroxidation. Hence, NO reduces ROS production by inhibiting

mitochondrial respiratory chain complexes[13].

NO activates sGC-GTP-cGMP signaling pathway to reduce liver cell apoptosis

NO derived from blood vessels can activate soluble guanylyl cyclase (sGC), catalyzing guanosine triphosphate (GTP) to produce cyclic 3′, 5′ guanosine monophosphate (cGMP). The protection of cGMP-dependent protein kinase (PKG) activated by cGMP results in the activation of PI3K and the phosphorylation of p38 MAPK, leading to the activation of vacuolar H^+-ATPases (V-ATPases), which lead to the extrusion of $[H^+]$ ($[H^+]i$) from the cytosol of hepatocytes into the extracellular environment, thereby resulting in inhibition of the H^+-driven Na^+/H^+ exchanger (NHE) and Na^+/HCO_3^- cotransporter (NHCT), with a consequent reduction in $[Na^+]$ ($[Na^+]i$) and protection from hepatocyte death[5]. Diao *et al*[11] observed that NO plays an important protective role in organ preservation by supplementing sufficient NO donors to enhance the NO/cGMP pathway.

NO regulates hepatic immune function

NO is also an important effector molecule that is involved in immune regulation and host innate and acquired immunity. NO inhibits proinflammatory cytokines, including TNF-α, IL-1β, IL-1α, and IL-12, which may induce the inflammatory cascade during HIRI. In addition, NO can decrease the number of T helper 1 (Th1) cells and promote the proliferation of Th2 cells, regulate leukocyte adhesion, and induce the generation of T regulatory (Treg) cells[14,15].

It has also been reported that excessive NO may paradoxically damage liver tissue by forming nitrogen peroxide, indicating that the dose of exogenous NO donors is vital to HIRI therapy.

ENOS CONTRIBUTES TO PROTECTIVE FUNCTIONS AGAINST HIRI

ENOS activation increases NO levels

Intracellular Ca^{2+} levels are the key factors that activate eNOS. During HIRI, Na^+/Ca^{2+} exchange protein on the cell membrane is activated directly or indirectly by the high concentration of Na^+, H^+, and PKC, leading to an increase in intracellular Ca^{2+}. Furthermore, with the liver cell membrane structure damaged, Ca^{2+} transports into the cellular membrane increased, and the endoplasmic reticulum and sarcoplasmic reticulum are also destroyed, which inhibits the function of the calcium pump to elevate the intracellular Ca^{2+} concentration. Meanwhile, stored intracellular Ca^{2+} is released. All these may lead to a higher intracellular Ca^{2+} concentration, which activates eNOS to produce more NO[16,17]. The basic low-dose NO catalyzed by eNOS could mitigate the hepatic microcirculation pressure caused by reperfusion.

ENOS/NO SIGNALING PATHWAYS PARTICIPATE IN ANTI-HIRI ACTIVITY

Akt-eNOS/NO pathway

PI3K is a heterodimer composed of the catalytic subunit p10 and regulatory subunit p85, and is also a lipid second messenger. PI3K can phosphorylate the serine/threonine of its downstream signal kinase Akt, which may further phosphorylate eNOS to promote an increase in endogenous NO generation. The protective effect of rHuEPO in IR injury is mediated *via* the activation of the PI3K/AKT/eNOS signaling pathway, at least in part, by increasing p-AKT and p-eNOS, which leads to the maintenance of an elevated level of NO[18]. It has been reported that IGL-1 solution results in better liver preservation and protection against HIRI by activating Akt and AMPK, which are concomitant with increased eNOS expression and nitrite/nitrate levels[19].

AMPK-eNOS/NO pathway

Zhang C reported that adiponectin (APN) can protect the liver from HIRI by reducing the inflammatory reaction and hepatocyte apoptosis, the process that likely involves the AMPK/eNOS pathway[20]. In addition, heparin cofactors II (HCII) potentiates hepatic vascular endothelial cell activity and the promotion of angiogenesis *via* an AMPK/eNOS signaling pathway to decrease vascular injury[21].

HIF-1α-eNOS/NO pathway

Adding trimetazidine, an anti-ischemia drug, to IGL-1 induces NO and eNOS activation. In normoxic reperfusion, the presence of NO favors hypoxia-inducible factor-1α (HIF-1α) accumulation, and also promotes the activation of other cytoprotective genes to reduce HIRI, such as heme-oxygenase-1. In addition, NO could reduce HIRI *via* the HIF-1α/NO pathway[22].

Other new pathways

Deregulated long noncoding RNA (LncRNAs) AK139328 is involved in HIRI. In the IR liver, the knockdown of AK139328 increases survival-signaling proteins including phosphorylated Akt (pAkt), glycogen synthase kinase 3 (pGSK3), and endothelial nitric oxide synthase (peNOS). Furthermore, the knockdown of AK139328 also reduces macrophage infiltration and inhibits NF-κB activity and inflammatory cytokine expression[23]. This could provide some new options for the diagnosis and treatment of liver diseases, such as surgery or transplantation.

Inhibition of eNOS overexpression to protect HIRI

The most current evidence supports the idea that the overexpression of eNOS is detrimental in the setting of hepatic IR[24]. Sanches SC found that during HIRI, the riboflavin infusion partially recovered hepatic GSH reserves and decreased eNOS/iNOS and NO levels in

the liver, and that riboflavin could have antioxidant and anti-inflammatory effects in the ischemic liver, protecting hepatocytes against IR injury[25].

Rosmarinic acid, which is a kind of water-soluble phenolic acid compound and a natural antioxidant, has many biological functions, such as antibacterial, antiviral, and anti-inflammatory effects, prevention of high calcium concentrations in the cell, and regulation of immune function. Also, it could inhibit eNOS overexpression in the liver, decrease eNOS/iNOS and NO levels in the liver, attenuate NF-κB activation, downregulate TNF-α and IL-1β gene expression, and exert anti-inflammatory and antioxidant effects in the ischemic liver, thereby protecting hepatocytes against IRI[26].

ROLE OF INOS IN HIRI

During sudden hepatic ischemic stress, upregulated iNOS in the liver produces a large quantity of NO as a response. However, up-regulating the expressions of iNOS gene and protein requires time[7].

iNOS aggravates HIRI

Some evidence suggests that eNOS can lead to "dysfunction" during oxidative stress, so production of a large amount of NO against IRI appears to be necessary for the expression of iNOS[27,28]. Hu *et al*[29] discovered that while the expression of iNOS mRNA peaked 3 h after hepatic reperfusion, the highest protein level appeared after 6 h. After 4 h of reperfusion, the increased iNOS mRNA transcription did not result in increased NO production, and this lack of increase may be linked to different degrees of tissue damage[30].

Inhibition of iNOS prevented liver from HIRI

Alpha lipoic acid (α-LA) has been shown to alleviate HIRI in rats. The underlying mechanism may be that α-LA inhibits the expression of the iNOS gene antisense-transcript, which is involved in iNOS mRNA stability. Therefore, there may be useful therapeutic effects associated with the suppression of iNOS induction involved in liver injury[31].

IL-1β and TNF-α are important proinflammatory cytokines[14]. The upregulation of IL-1β receptors accelerates the iNOS transcription process, but the mechanism involved in its downstream signaling pathway is unknown[32]. Blocking IL-1 receptors may be a way to alleviate HIRI. Although it is uncertain whether the reduction of IL-1 receptors is related to iNOS transcription, it does show that iNOS is involved in the process[33].

CONCLUSION

NO plays a complicated role during HIRI. NO can inhibit the expression of p53 gene and the aggregation of proinflammatory cytokines and chemokines, reduce ROS by inhibiting the mitochondrial respiratory chain, participate in hepatic immune modulation, inhibit the inflammatory cascade, and exhibit anti-inflammatory properties. In addition, as the first messenger, NO activated the NO/cGMP pathway to inhibit $[Na^+]i$ from entering the cells, thereby helping to maintain hepatic cell integrity. Conversely, excessive NO in serum can aggravate liver injury. Elevating NO levels appropriately, such as by applying exogenous NO donors or increasing NO availability in the liver, may be a good way to prevent and treat HIRI. There are two ways to activate eNOS which can promote an increase in endogenous NO generation to protect liver tissue from HIRI. One is elevating Ca^{2+} levels in cells or phosphorylating the active site of eNOS gene, and the other is knocking out gene AK139328. Both can protect the liver from HIRI. But excessive NO levels derived from eNOS are detrimental to the liver. iNOS has a synergistic effect with some inflammatory mediators, which cause cellular swelling and apoptosis. Excessive NO derived from iNOS plays a protective role in the late period of HIRI. Thus, adverse effects of NO should be avoided, and its positive effects in the clinical treatment of diseases associated with HIRI should be recognized.

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ORIGINAL ARTICLE

Basic Study

Berberine displays antitumor activity in esophageal cancer cells *in vitro*

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Abstract

AIM

To investigate the effects of berberine on esophageal cancer (EC) cells and its molecular mechanisms.

METHODS

Human esophageal squamous cell carcinoma cell line KYSE-70 and esophageal adenocarcinoma cell line SKGT4 were used. The effects of berberine on cell proliferation were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For cell cycle progression, KYSE-70 cells were stained with propidium iodide (PI) staining buffer (10 mg/mL PI and 100 mg/mL RNase A) for 30 min and cell cycle was analyzed using a BD FACSCalibur flow cytometer. For apoptosis assay, cells were stained with an Annexin V-FITC/PI apoptosis detection kit. The rate of apoptotic cells was analyzed using a dual laser flow cytometer and estimated using BD ModFit software. Levels of proteins related to cell cycle and apoptosis were examined by western blotting.

RESULTS

Berberine treatment resulted in growth inhibition of KYSE-70 and SKGT4 cells in a dose-dependent and time-dependent manner. KYSE-70 cells were more

susceptible to the inhibitory activities of berberine than SKGT4 cells were. In KYSE-70 cells treated with 50 μmol/L berberine for 48 h, the number of cells in G_2/M phase (25.94% ± 5.01%) was significantly higher than that in the control group (9.77% ± 1.28%, $P < 0.01$), and berberine treatment resulted in p21 up-regulation in KYSE-70 cells. Flow cytometric analyses showed that berberine significantly augmented the KYSE-70 apoptotic population at 12 and 24 h post-treatment, when compared with control cells (0.83% *vs* 43.78% at 12 h, $P < 0.05$; 0.15% *vs* 81.86% at 24 h, $P < 0.01$), and berberine-induced apoptotic effect was stronger at 24 h compared with 12 h. Western blotting showed that berberine inhibited the phosphorylation of Akt, mammalian target of rapamycin and p70S6K, and enhanced AMP-activated protein kinase phosphorylation in a sustained manner.

CONCLUSION

Berberine is an inhibitor of human EC cell growth and could be considered as a potential drug for the treatment of EC patients.

Key words: Berberine; Esophageal cancer; Antitumor activity; Proliferation; Cell cycle; Apoptosis

Core tip: Initial diagnosis of many esophageal cancer (EC) patients is made at an advanced stage of the disease, making surgery an undesirable option. Although advances in chemotherapy have been achieved, serious adverse effects usually limit clinical application. Exploring non-invasive strategies to prevent the growth of EC is urgently needed. The current research showed that berberine is an inhibitor of human EC cell growth and could be considered as a potential source of drugs for the treatment of EC patients.

Jiang SX, Qi B, Yao WJ, Gu CW, Wei XF, Zhao Y, Liu YZ, Zhao BS. Berberine displays antitumor activity in esophageal cancer cells *in vitro*. *World J Gastroenterol* 2017; 23(14): 2511-2518 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i14/2511.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i14.2511

INTRODUCTION

Esophageal cancer (EC) is the sixth most common malignant gastrointestinal carcinoma worldwide. More than 50% of the global incidence of EC is in China[1]. A report published in 2016 shows that there are an 477900 and 375000 estimated new EC cases and deaths, respectively, in China[2]. Histologically, EC is divided into two major types: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). More than 90% of EC in China is ESCC. Although advances have been achieved in surgery and chemotherapy, the 5-year survival rate of EC in China is only 19.9%[3]. Esophagostomy is so far the only potentially curative approach for EC, but many patients are at an advanced stage of disease during initial diagnosis, thus ruling them out from surgery. Therefore, there is a critical need to develop alternative and novel approaches in EC therapy.

Berberine is a quaternary ammonium salt derived from Ranunculaceae and Papaveraceae families of plants. Apart from a broad range of bioactivities, such as anti-inflammatory, antibacterial and antidiabetic actions, accumulating studies have revealed that berberine exhibits antitumor properties by interfering with the multiple features of tumorigenesis and tumor development[4]. The antitumor activity of berberine is mainly mediated through the inhibition of cancer cell proliferation by inducing cell cycle arrest at the G1 or G2/M phases and initiation of apoptosis[5,6]. Previous studies have reported that berberine inhibits the phosphatidylinositol 3-kinase (PI3K)/Akt /mammalian target of rapamycin (mTOR)[7,8] signaling cascades to inhibit cell proliferation in various cell lines derived from breast, lung, colon and liver cancer[9-12]. Berberine also activates AMP-activated protein kinase (AMPK), a major regulator of metabolic pathways, subsequently inhibiting mTOR, a downstream target of AMPK[12,13].

Although berberine possesses numerous anticancer activities in various cells, the effect of berberine on EC growth and its mechanism of action have not yet been fully elucidated. In this study, we reported that berberine inhibited EC cell growth by promoting cell cycle arrest at G2/M phase as well as apoptosis. The Akt, mTOR/p70S6K and AMPK signaling pathways were involved in the antitumor activity of berberine on EC.

MATERIALS AND METHODS

Reagents

Berberine hydrochloride was obtained from Ye-Yuan (Shanghai, China). 3-(4,5-dimethylthia-zol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), propidium iodide (PI) cell cycle assay kit, Annexin V-FITC/PI apoptosis detection kit and western blot analysis ECL were purchased from Beyotime (Jiangsu, China). RPMI 1640 and fetal bovine serum (FBS) were obtained from Thermo Fisher Scientific (Waltham, MA, United States). All primary antibodies, including against p21, Akt, p-Akt (Ser473), mTOR, p-mTOR (Ser2448), p70S6K, p-p70S6K (Thr389), AMPK, p-AMPK (Thr172) and β-actin, were from Cell Signaling Technology (Danvers, MA, United States). All other common chemicals and buffers were from Boster (Wuhan, China).

Cell culture and maintenance

Human ESCC cell line KYSE-70 and EAC cell line

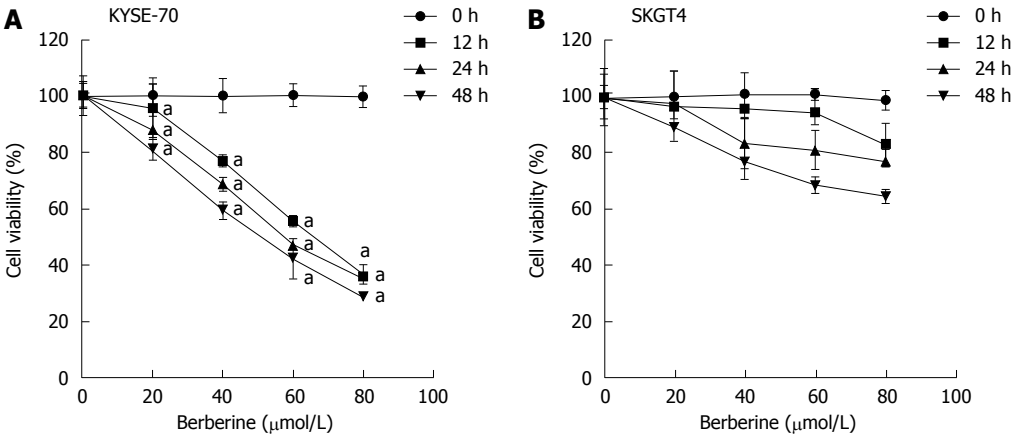

Figure 1 Effects of berberine on viability of esophageal cancer cells. A, B: KYSE-70 (A) and SKG4 (B) cells were treated with berberine (0, 20, 40, 60 and 80 μmol/L) for 12, 24 and 48 h and the number of viable cells was measured by MTT assay. Data are expressed as mean ± SD of three experiments. $^{a}P < 0.05$ *vs* controls.

SKGT4 were purchased from Kebai Technology (Nanjing, China). The culture medium for both cell lines was RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were incubated in a humidified atmosphere with 5% CO_2 at 37 ℃.

Cell viability assay

Cell viability was measured by MTT assay. KYSE-70 (10^4/well) and SKGT4 (5000/well) were seeded in 96-well culture plates and incubated overnight at 37 ℃ in a humidified 5% CO_2 incubator. On the following day, cells were treated with berberine hydrochloride at indicated concentrations for indicated durations. Then, 10 μL MTT dye was added to each well at a final concentration of 5 mg/mL. For an additional 4 h after incubation, blue MTT formazan crystals were dissolved in 100 μL/well of DMSO. The absorbance at 562 nm was measured on a Multiskan Spectrum microplate reader (Thermo Fisher Scientific). Cell viability was calculated by dividing the OD of samples by the OD of the control group. All experiments were repeated three times.

Flow cytometric analyses of cell cycle and apoptosis

KYSE-70 cells (8 × 10^4/well) were seeded in six-well plates in complete culture medium. After incubating for 12 h, cells were treated with berberine hydrochloride (50 μmol/L). Cells were harvested separately at 12 and 24 h later, and immediately fixed with 75% ethanol. For the cell cycle progression analysis, cells were stained with PI staining buffer (10 mg/mL PI and 100 mg/mL RNase A) for 30 min, and fluorescence intensity was measured by BD FACSCalibur (BD Biosciences**,** San Jose, CA**,** United States). For apoptosis analysis, cells were stained with the Annexin V-FITC/PI apoptosis detection kit. The rate of apoptotic cells was analyzed using a dual laser flow cytometer and estimated using the ModFit software (BD Biosciences).

Western blot analysis

Cell lysates were prepared with RIPA lysis buffer (50 mM Tris-HCl, 150 mmol/L NaCl, 0.1% SDS, 1% NP40, 0.5% sodium deoxycholate, 1 mmol/L phenylmethylsulfonyl fluoride, 100 μmol/L leupeptin, and 2 μg/mL aprotinin, pH 8.0). Protein extract (20 μg) was subjected to SDS-PAGE and transferred onto nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, United States). After blocking with 5% nonfat dry milk, membranes were incubated at 4 ℃ overnight with each of the following primary antibodies: p21, pAKT (Ser473), AKT, p-mTOR (Ser2448), mTOR, pp70S6K (Thr389), p70S6K, p-AMPK (Thr172), AMPK (all 1:1000 dilution) and β-actin. Membranes were washed with phosphate buffered saline plus Tween (PBST) buffer and incubated with horseradish peroxidase-conjugated secondary antibodies. After incubation, the membranes were washed three times with PBST and immersed in a SuperSignal West Pico Chemiluminescent Substrate from the detection kit (Thermo Fisher Scientific). Chemiluminescent detection of western blots was performed using an Amersham Imager 600 System (GE Healthcare Bio-Sciences, Pittsburgh, PA, United States).

Statistical analysis

Data were analyzed using Student's *t*-test, and all data were expressed as mean ± SE of the mean. $P < 0.05$ was considered statistically significant.

RESULTS

Growth suppressive effect of berberine on human EC cells

To examine the biological consequences of berberine, we first examined its effect on the proliferation of ESCC and EAC cells. We observed that berberine significantly suppressed KYSE-70 proliferation after treatment with different concentrations (20, 40, 60

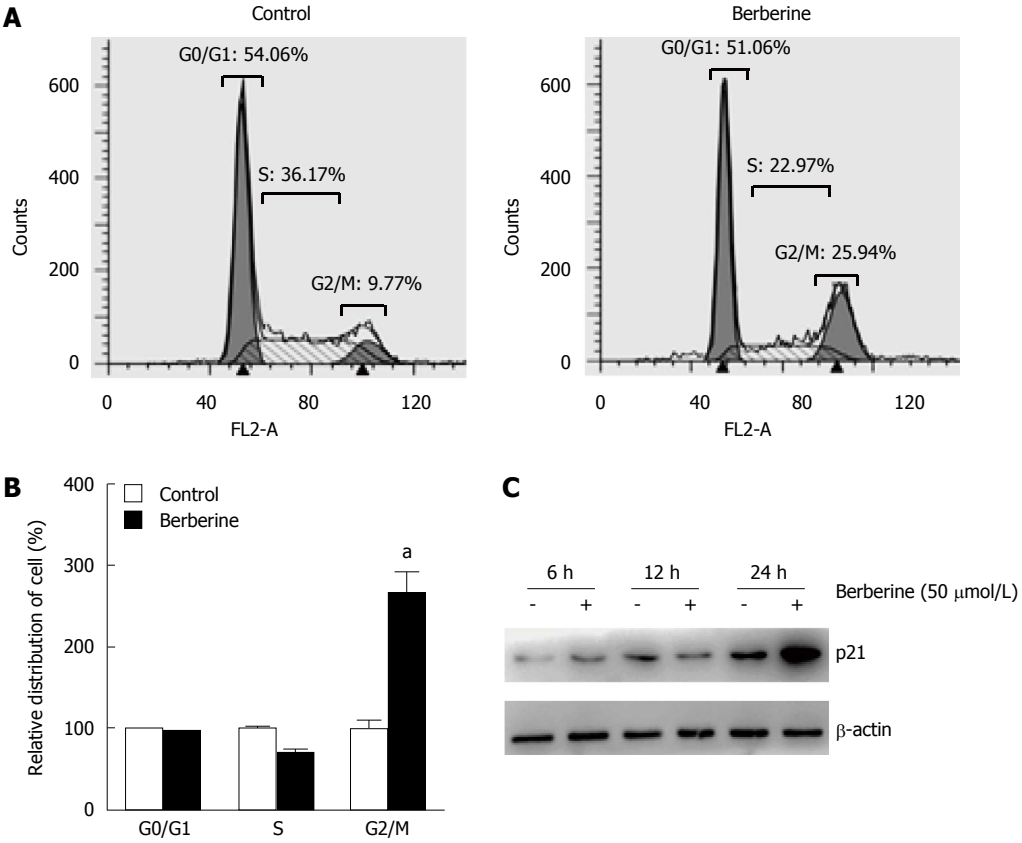

Figure 2 Berberine treatment induced cell cycle arrest in G2/M phase. A: Flow cytometry analysis of proliferating KYSE-70 cells at 48 h after administration of 50 μmol/L berberine; B: Relative percentages of berberine-treated cells to control cells in different cell cycle phases are shown as the mean ± SE of three independent experiments. [a]*P* < 0.05 *vs* controls; C: Protein expression level of p21 in KYSE-70 cells was examined after berberine administration at 6, 12 and 24 h.

and 80 μmol/L) at all tested time points (12, 24 and 48 h) (Figure 1A). Berberine had significantly suppressive effects on SKGT4 cell proliferation when tested at 24 and 48 h after treatment with berberine at 20, 40, 60 or 80 μmol/L. At the 12-h time point, berberine did not significantly inhibit SKGT4 cell proliferation until the concentration reached 80 μmol/L (Figure 1B). Upon comparison of the proliferation inhibitory effects of berberine against the two cell lines, KYSE-70 was more sensitive than SKGT4 to the dose-dependent and time-dependent suppressive effects of berberine. Therefore, we focused further on KYSE-70 cells in the following experiments.

Cell cycle arrest effect of berberine on human EC cells

To clarify whether impairment of cell cycle involved in the reduction of KYSE-70 growth was induced by berberine, KYSE-70 cells were treated with 50 μmol/L berberine for 48 h, stained with PI, and subjected to cell cycle progression analysis using flow cytometry. As shown in Figure 2A and B, when compared with the controls, it is evident that the fraction of G2/M cells was increased after berberine treatment (9.77% *vs* 25.94%, $P < 0.01$), whereas in parallel, we did not observe significant changes in cell numbers in G0/G1 phase (54.06% *vs* 51.06%). To explore further the molecular signals involved in berberine-induced G2/M phase arrest, Western blot analysis was used to determine the expression of p21; a key cell cycle negatively regulated protein. As shown in Figure 2C, after application of berberine at 50 μmol/L for 24 h, p21 level was increased. This indicates that berberine-induced cell cycle arrest at G2/M phase in KYSE-70 cells is mediated through p21 down-regulation.

Apoptotic effect of berberine on EC cells

To evaluate whether the antiproliferative activity of berberine was related to its apoptotic effect, KYSE-70 cells were treated with 50 μmol/L berberine, and flow cytometric analyses were performed by double staining with Annexin-V FITC/PI. As shown in Figure 3, berberine significantly increased KYSE-70 cell apoptosis (0.15% *vs* 43.73% at 12 h, $P < 0.05$; 0.83% *vs* 81.86% at 24 h, $P < 0.05$). We next evaluated the effect of berberine on KYSE-70 cell morphology. Phase contrast imaging (Figure 4) showed that untreated control KYSE-70 cells were epithelial-like adherent cells, with a flat and polygonal shape, that grew homogeneously and showed strong refraction. When treated with berberine, the cells showed reduced refraction and shrunk to a round shape. The treated cells grew in a scattered way, resulting in loss of intercellular conjunction. Consistent with the data in Figure 1, phase contrast imaging showed

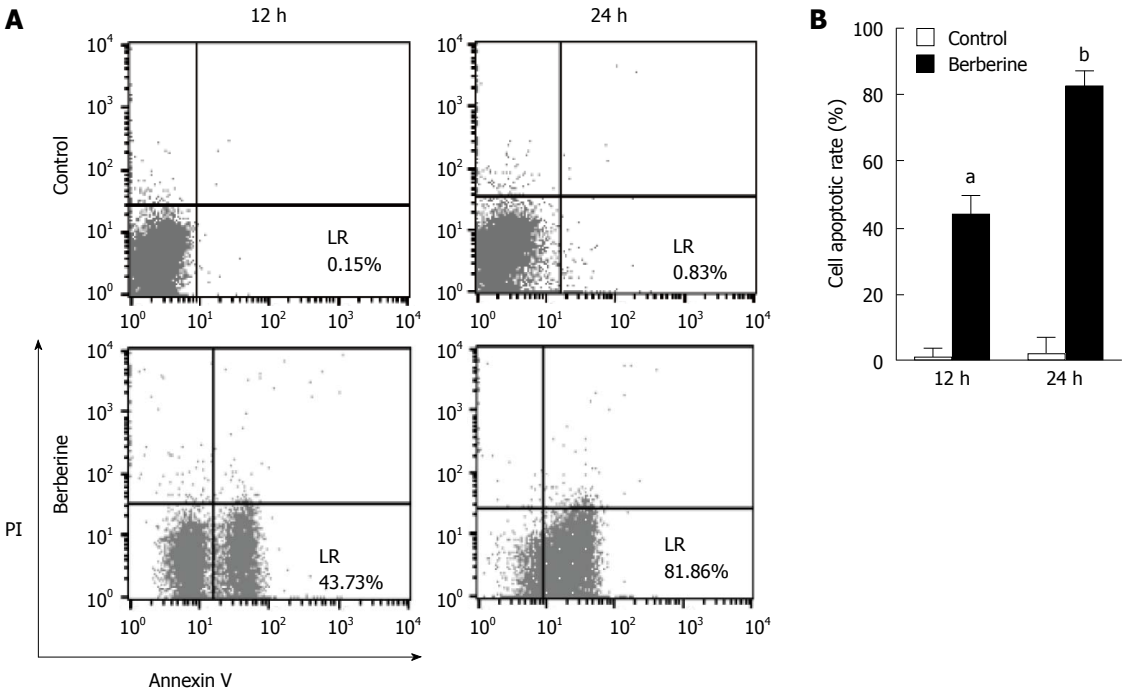

Figure 3 Berberine promotes apoptosis in KYSE-70 cells. A: KYSE-70 cells were treated with 50 μmol/L berberine for 12 and 24 h. Apoptotic rates were measured using flow cytometry; B: Apoptotic cell values are expressed as mean ± SE of three experiments. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* controls.

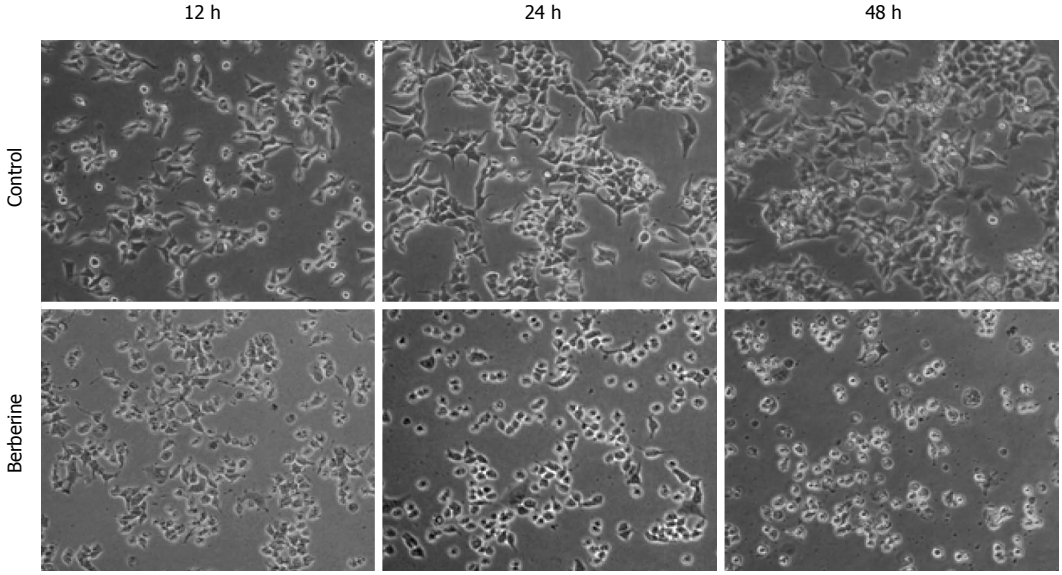

Figure 4 Berberine treatment induced morphological changes of KYSE-70 cells. Control cells and 50 μmol/L berberine-treated cells were observed under a phase contrast microscope at 12, 24 and 48 h after treatment. Bar represents all images equal to 200 μmol/L.

that berberine suppressed proliferation and promoted apoptosis.

Berberine inhibited cell proliferation through Akt/mTOR/p70S6k and AMPK signaling pathways

Previous studies have indicated that inhibiting Akt/mTOR/p70S6K signaling and activating AMPK contribute to berberine-induced loss of cell viability[9,14]. To address whether these signaling molecules are related to the biological consequences of berberine in KYSE-70 cells, western blot analyses were performed to examine the phosphorylation levels of these signaling molecules. Cells were treated with 50 μmol/L berberine for 6, 12 or 24 h, in comparison with control cells at each time point. Berberine markedly reduced phosphorylation of Akt at Ser473, mTOR at Ser2448 and p70S6K at Thr389, starting as early as 6 h after treatment and sustaining a reduced level for 24 h. Berberine clearly enhanced AMPK phosphorylation at Thr172 after 6 h treatment, and maintained increasing levels for 24

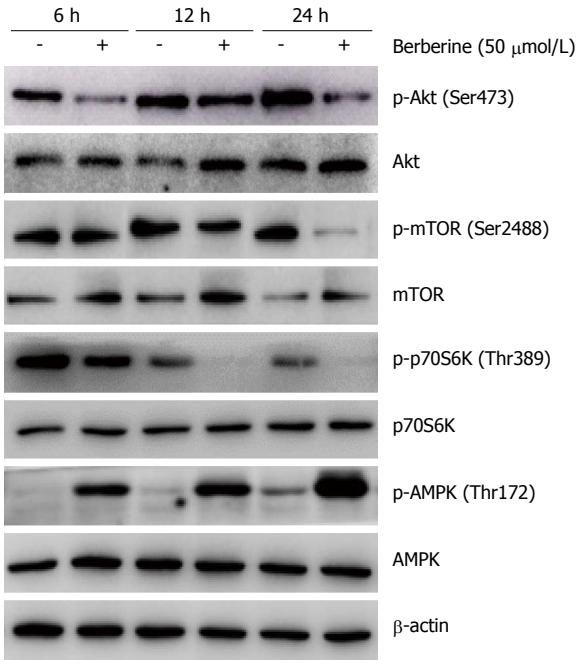

Figure 5 Effects of berberine on AMPK and AKT/mTOR/p70S6K activities. KYSE-70 cells were treated with 50 μmol/L berberine for 6, 12 and 24 h, and protein expressions of p-AKT, AKT, p-mTOR, mTOR, p-p70S6K, p70S6K, p-AMPK and AMPK were analyzed by western blotting.

h after treatment. These data suggest that inhibition of Akt-mTOR/p70S6K and activation of AMPK are important targets of berberine activity (Figure 5).

DISCUSSION

The low survival rate of EC patients is associated with poor prognosis of the disease and advanced stage at initial diagnosis, thus making surgery an undesirable option. Although advances have been achieved in chemotherapy, the serious adverse effects usually limit clinical application[14]. Therefore, there is a critical need to develop non-invasive strategies to confine the growth or prevent the occurrence of EC.

Compounds derived from plants have been identified as an important source of anticancer therapies and have played a vital role in the prevention and treatment of cancer because of their availability and low toxicity when compared with chemotherapy[15,16]. A compound derived from an alkaloid-containing plant, berberine, has been shown to possess numerous anticancer activities in various cells by interfering with the multiple aspects of tumorigenesis and tumor progression[9-12]. Despite this, it has remained unconfirmed whether berberine exerts an antitumor effect against EC. In the present study, we found that berberine induced strong growth inhibition of the human ESCC cell line KYSE-70 and EAC cell line SKGT4 in a dose-dependent and time-dependent manner. KYSE-70 cells were more susceptible than SKGT4 cells to the inhibitory effects of berberine. Our findings indicate that berberine is a potent inhibitor of human EC cell growth and could be considered as a potential source of drugs for the treatment of EC patients.

Cell cycle arrest and apoptosis are closely linked to cell proliferation in mammalian cells[17]. The major regulatory mechanism of cell growth, the cell cycle dictates the timing of DNA synthesis, and is divided into four distinct phases: M phase (chromosome segregation and mitosis), G1 phase (before DNA replication), S phase (DNA replication) and G2 phase (before mitosis). The cell cycle process includes mechanisms to warrant error amendment, and if not, the cells commit apoptosis, which is one of the most important contributors to the suppression of malignant transformation and elimination of tumors. Control of cell numbers is determined by a complicated balance of cell proliferation and death.

Previous studies have shown that berberine induces cell cycle arrest in various human cancer cells[5,6]. To determine whether berberine prompts cell cycle arrest of KYSE-70 cells, the cell cycle distribution was analyzed by flow cytometry after application of berberine. Our results demonstrated that berberine significantly blocked KYSE-70 cells at the G2/M phase of the cell cycle, suggesting that berberine inhibits KYSE-70 cell proliferation by inducing G2/M cell cycle arrest. These data are in agreement with previous studies in human breast cancer cells and liver cancer cells[6,11]. Appropriate control over cell cycle progression depends on many factors, such as cyclin-dependent kinase inhibitor p21 facilitating cell cycle arrest in response to a variety of stimuli. Our results showed that berberine augmented p21 level in KYSE-70 cells, indicating that berberine-induced cell cycle arrest in G2/M phase may be through regulation of cell cycle protein p21.

The PI3K/Akt/mTOR signaling pathway plays a crucial role in controlling cell proliferation and apoptosis[18]. Constitutive activation of this pathway is considered to be important in cell growth and homeostasis[19]. Specifically, activated mTOR directly phosphorylates many downstream targets including p70S6K to promote protein synthesis[20]. As a major regulator of cellular energy metabolism, AMPK is a negative regulator of the mTOR pathway[20,21]. Berberine regulation of cell proliferation and survival has been shown to involve Akt, mTOR/p70S6K and AMPK signaling pathways[10-12]. Our results showed that berberine treatment inhibited the phosphorylation of Akt and mTOR, as well as mTOR downstream target p70S6K, but enhanced the phosphorylation of AMPK. A previous study reported that, in breast cells, berberine transiently activated AMPK and inhibited AKT, but did not inhibit mTOR activity[22]. Our results showed that treatment with berberine induced sustained alterations (6-24 h) of increased levels of AKT and mTOR phosphorylation in KYSE-70 cells or increased level of AMPK phosphorylation in KYSE-70 cells. These results suggest that berberine alters Akt, mTOR and AMPK

activity in an individual cell-dependent manner.

In conclusion, it is suggested that berberine inhibits EC cell growth by promoting cell cycle arrest at G2 phase and the apoptotic process. The Akt, mTOR/p70S6K and AMPK signaling pathways are involved in the antitumor activity of berberine on EC. We have shown that berberine is an inhibitor of human EC cell growth and could be considered as a potential source of drugs for the treatment of EC patients.

COMMENTS

Background

The initial diagnosis of many esophageal cancer (EC) patients is at an advanced stage, making surgery an undesirable option. Although advances have been achieved in chemotherapy, serious adverse effects usually limit its clinical application. Therefore, there is an urgent need to find non-invasive strategies to confine the growth or prevent the occurrence of EC. Berberine, a compound derived from an alkaloid-containing plant, has been shown to possess numerous anticancer activities in various cells by interfering with the multiple aspects of tumorigenesis and tumor progression. Despite this, it has remained unconfirmed whether berberine exerts antitumor effects against EC.

Research frontiers

Accumulating studies have revealed that berberine exhibits antitumor activity by interfering with the multiple features of tumorigenesis and tumor development.

Innovations and breakthroughs

This study revealed that berberine inhibited EC cell growth by promoting cell cycle arrest at G2/M phase and the apoptosis process. Human esophageal squamous cell carcinoma cells were more susceptible to the inhibitory activity of berberine than human esophageal adenocarcinoma cells. Inhibition of Akt, mTOR/p70S6K and activated AMPK signaling pathways was involved in the antitumor activity of berberine on EC.

Terminology

Berberine is a quaternary ammonium salt derived from Ranunculaceae and Papaveraceae families of plants. Apart from a broad range of bioactivities that includes anti-inflammatory, antibacterial and antidiabetic activity, berberine has been shown to have antitumor activity, which it exerts by interfering with the multiple features of tumorigenesis and tumor development.

Peer-review

EC is still a cancer with poor prognosis. The testing of chemosensitivity of cancer cells in the esophagus to berberine is important. This enables classification of the condition for selected patients with esophageal cancer to be treated. Berberine can provide treatment options for adjuvant or preoperative treatment.

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ORIGINAL ARTICLE

Case Control Study

Clinical utility of the platelet-lymphocyte ratio as a predictor of postoperative complications after radical gastrectomy for clinical T2-4 gastric cancer

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Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of the Nagoya University.

Informed consent statement: This study conforms to the ethical guidelines of the World Medical Association Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects, and written informed consent for the use of clinical data were obtained from all patients.

Conflict-of-interest statement: The authors declare that they have no conflicts of interest.

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Abstract

AIM

To identify simple and sensitive markers for post-operative complications after gastrectomy, the predictive values were compared among candidate preoperative factors.

METHODS

Three-hundred and twelve patients with previously untreated clinical T2-4 gastric cancer who underwent a D2 standard gastrectomy (distal gastrectomy or total gastrectomy) were included in the analysis.

Correlations between 21 parameters that can be determined by preoperative routine blood tests and clinically relevant postoperative complications (grade Ⅱ or higher according to the Clavien-Dindo classification) were evaluated. The optimal cutoff values and clinical significance of the selected markers were further evaluated by subgroup analyses according to age, body mass index, operative procedure and clinical disease stage.

RESULTS

Sixty-six patients (21.1%) experienced grade Ⅱ or higher postoperative complications. The platelet-lymphocyte ratio (PLR, total lymphocyte count/platelet count × 100) exhibited the highest area under the curve value (0.639) for predicting postoperative complications among the 21 parameters, and the optimal cutoff value was determined to be 0.71 (sensitivity = 70%, specificity = 56%). In the univariate analysis, the odds ratio of a low PLR for the occurrence of postoperative complications was 2.94 (95%CI: 1.66-5.35, $P < 0.001$), and a multivariate binomial logistic analysis involving other potential risk factors identified a low PLR as an independent risk factor for postoperative complications (OR = 3.32, 95%CI: 1.82-6.25, $P < 0.001$). In subgroups classified according to age, body mass index, operative procedure and clinical disease stage, the low PLR group exhibited an increased incidence of postoperative complications.

CONCLUSION

The preoperative PLR is a simple and useful predictor of complications after curative gastrectomy in patients with clinical T2-4 gastric cancer.

Key words: Gastric cancer; Gastrectomy; Platelet-lymphocyte ratio; Postoperative complication; Prediction

Core tip: The prediction of postoperative complications is important for providing appropriate perioperative management. In the present study, the predictive values for postoperative complications after gastrectomy with systemic lymphadenectomy for gastric cancer were compared among candidate preoperative factors to identify a simple and sensitive marker. Our results indicated that the preoperative platelet-lymphocyte ratio is a simple and useful predictor for complications after curative gastrectomy in patients with gastric cancer.

Inaoka K, Kanda M, Uda H, Tanaka Y, Tanaka C, Kobayashi D, Takami H, Iwata N, Hayashi M, Niwa Y, Yamada S, Fujii T, Sugimoto H, Murotani K, Fujiwara M, Kodera Y. Clinical utility of the platelet-lymphocyte ratio as a predictor of postoperative complications after radical gastrectomy for clinical T2-4 gastric cancer. *World J Gastroenterol* 2017; 23(14): 2519-2526 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i14/2519.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i14.2519

INTRODUCTION

Gastrectomy with systemic lymphadenectomy is the mainstay of treatment for resectable gastric cancer (GC)[1,2]. Despite advances in surgical techniques and devices, some patients who undergo the procedure experience clinically relevant postoperative complications, such as anastomotic leakage and intraabdominal abscess, leading to a protracted recovery period, delayed administration of adjuvant chemotherapy and impaired quality of life[3,4]. Risk management is an increasingly important healthcare issue. Developing a prediction tool based exclusively on preoperatively determined factors to identify patients most at risk of postoperative complications enables surgeons to provide appropriate informed consent information and perioperative management, ultimately minimizing the medical cost burden[5,6].

Increasing evidence indicates that multiple factors influence local infection control and the process of wound healing[7]. Accordingly, numerous reports on predictive factors for postoperative complications, including inflammatory, immunological, nutritional, coagulation and organ functional indicators, have been published[8-11]. However, limited information from cross-comparisons of these factors is available for patients who undergo a D2 gastrectomy for GC.

The aim of the present study was to compare the predictive values for postoperative complications after gastrectomy among candidate preoperative factors based on routinely obtained laboratory data. After the factor that demonstrated the highest predictive value was determined, the optimal cutoff value and its clinical significance were evaluated.

MATERIALS AND METHODS

Ethics

This study conforms to the ethical guidelines of the World Medical Association Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects, and written informed consent for surgery and the use of clinical data were obtained from all patients as required by the Institutional Review Board of Nagoya University.

Patients, surgical procedure and perioperative treatment

A total of 1194 patients underwent a gastrectomy for GC at the Department of Gastroenterological Surgery, Nagoya University between 1999 and 2016. We retrospectively analyzed the data of 312 patients according to the following inclusion criteria: no preoperative treatment; clinical T2-4 (advanced) GC according to the TNM Classification of Malignant

Table 1 Demographic and preoperative clinical characteristics of 312 patients

Variables	Number of patients
Age, median (range)	66 (20-96)
Sex	
Male	233
Female	79
Diabetes mellitus	
Absent	273
Present	39
Cardiac comorbidities	
Absent	217
Present	95
Pulmonary comorbidities	
Absent	292
Present	20
Preoperative symptoms	
Absent	172
Present	140
Preoperative body mass index, mean ± SD	21.9 ± 3.2
Tumor location	
Entire	8
Upper third	68
Middle third	120
Lower third	116
Tumor size (mm)	
< 50	176
⩾ 50	136
UICC cT factor	
cT1	0
cT2	150
cT3	95
cT4	67
UICC cN factor	
cN0	157
cN1	93
cN2	53
cN3	9
UICC clinical stage	
ⅠB	98
ⅡA	71
ⅡB	69
ⅢA	55
ⅢB	14
ⅢC	5

UICC: Union for International Cancer Control.

Tumours, 7th Edition[12]; D2 gastrectomy (distal gastrectomy or total gastrectomy) performed according to the Japanese Gastric Cancer Treatment Guidelines[13]; no combined resection of other organs except for the spleen and gallbladder; R0 gastrectomy performed and sufficient data for analysis. The choice of the reconstruction method was at the surgeon's discretion. A first-generation cephem-based antibiotic was administered immediately before surgery and every 3 h during surgery. Oral intake was routinely started on postoperative day 1 if no obvious problems were found. Percutaneous drainage or the replacement of drainage tubes was performed when signs of inadequate drainage were noted by computed tomography or ultrasound scans. Clinically relevant postoperative complications were defined as those of grade Ⅱ or higher according to the Clavien-Dindo classification[14].

Study parameters

Blood tests were routinely performed two days before surgery. Data were collected retrospectively from the medical records focusing on five categories: blood cell count, coagulation, nutrition, renal function and inflammation. The parameters investigated as candidate predictive factors for postoperative complications, which can be rapidly measured in every hospital, included the following: white blood cell count, neutrophil count, total lymphocyte count (TLC), hemoglobin concentration, platelet count, prothrombin time, activated partial thromboplastin time, fibrinogen, total protein, albumin, cholinesterase, total cholesterol, urea nitrogen, creatinine and C-reactive protein (CRP). In addition, some simple indices were employed as candidate indicators: neutrophil-lymphocyte ratio (NLR = neutrophil count/TLC), platelet-neutrophil ratio (PNR = neutrophil count/platelet count × 100), platelet-lymphocyte ratio (PLR = TLC/platelet count × 100), Onodera's PNI (PNI = 10 × albumin g/dL + 0.005 × TLC/mm^3), Glasgow prognostic score (GPS) and the modified GPS[7,8,15].

Subgroup analyses

Subgroup analyses according to age, body mass index (BMI), operative procedure and clinical disease stage were performed to evaluate the correlations between the selected predictive factors and the incidence of clinically relevant postoperative complications.

Statistical analysis

A receiver operating characteristic (ROC) curve analysis was employed to calculate the area under the curve (AUC) and the sensitivity and specificity of the indicated variables to predict postoperative complications. The optimal cutoff value was determined using the Youden index[16]. The qualitative χ^2 test and quantitative Mann-Whitney test were used to compare the two groups. Multivariable analysis was performed to identify independent risk factors for postoperative complications using binomial logistic analysis, and variables with a value of $P < 0.05$ were included as covariates in the final model. All statistical analyses were performed using JMP 10 software (SAS Institute Inc., NC, United States). A statistically significant difference was indicated by a P-value < 0.05.

RESULTS

Patient characteristics

The demographic and preoperative characteristics of the 312 patients are presented in Table 1. The median age was 66 years, and the male-to female ratio was 233:79. With respect to preoperative staging, 98, 71, 69, 55, 14 and 5 patients were classified as clinical TNM stages ⅠB, ⅡA, ⅡB, ⅢA, ⅢB and ⅢC, respectively.

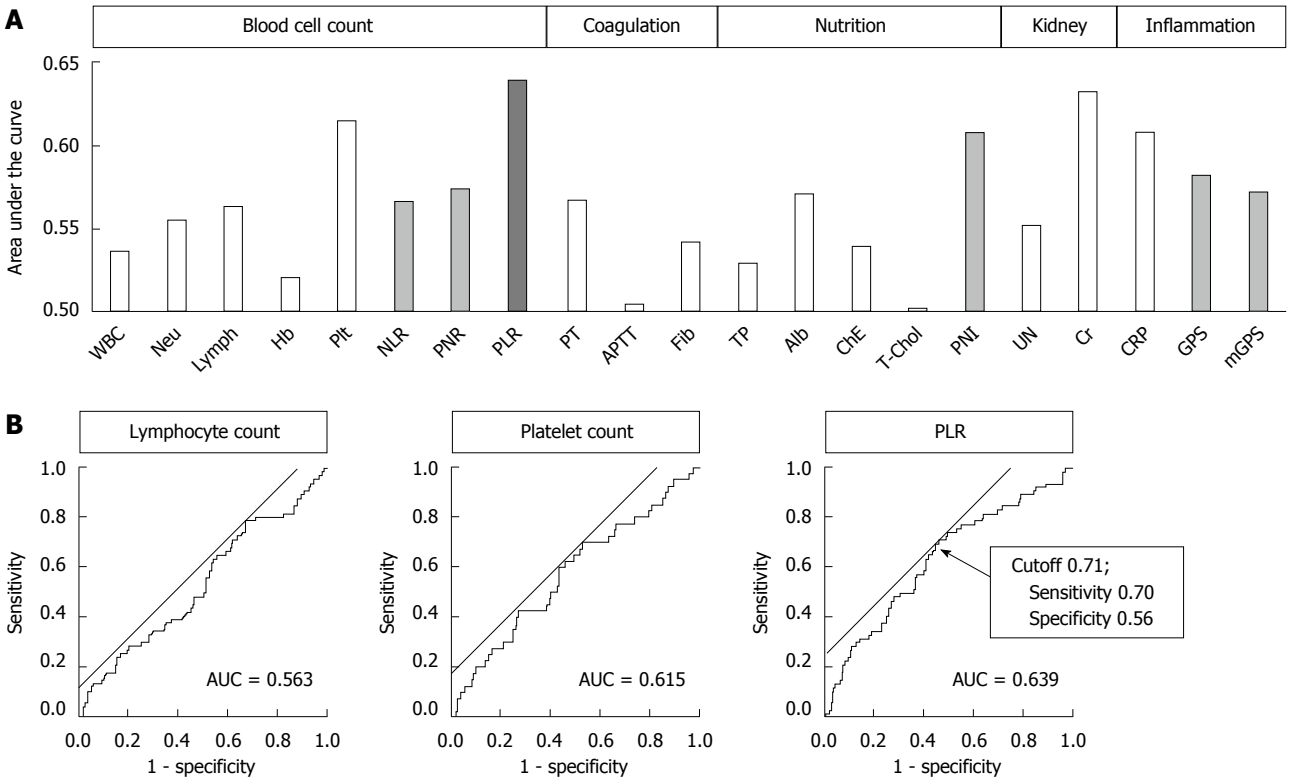

Figure 1 Comparison of the predictive value of potential indicators. A: Area under the curve values of potential predictors; B: Predictive values of lymphocyte count, platelet count and PLR for postoperative complications evaluated by receiver operating characteristic curve analysis. PLR: Platelet-lymphocyte ratio.

Comparison of predictive values among the candidate parameters

In total, 66 patients (21.1%) had grade Ⅱ or higher postoperative complications. In terms of the types of complications, the cumulative numbers of patients who experienced anastomotic leakage, leakage of pancreatic fluids, intraabdominal abscess and bowel obstruction were 20 (6.4%), 12 (3.8%), 19 (6.1%) and 6 (1.9%), respectively. When the AUC value, which indicates the power to predict postoperative complications, of the 21 parameters was analyzed, the PLR demonstrated the highest AUC value (0.639) (Figure 1A). This value was greater than the values of the PLR components TLC (AUC 0.563) and platelet count (AUC 0.615) (Figure 1B). The optimal cutoff value for predicting complications using the PLR was set at 0.71 (sensitivity = 70%, specificity = 56%) (Figure 1B). The AUC values of NLR, PNR, PNI and GPS were 0.566 (cutoff = 3.06), 0.574 (cutoff = 1.36), 0.608 (cutoff = 47.0) and 0.582 (cutoff = 1), respectively (Figure 1A).

Predictive value of the PLR

The median PLR was 0.71 (range 0.23-3.97), which is identical to the proposed cutoff. Using the cutoff indicated by the ROC curve analysis, we further evaluated the clinical significance of the PLR. Patients were categorized into two groups, the high (n = 158) and low (n = 154) PLR groups, using a PLR cutoff of 0.71. Compared to the high PLR group, patients in the low PLR group were associated with increased prevalence of pulmonary comorbidities, larger macroscopic tumor size and more frequent postoperative complications (Table 2). However, no significant differences were observed between the two groups regarding age, sex, preoperative BMI, clinical stage, type of gastrectomy, operative time, intraoperative blood loss and number of dissected lymph nodes (Table 2). In the univariate analysis, the odds ratio of a low PLR for postoperative complications was 2.94 (95%CI: 1.66-5.35, P < 0.001; Table 3). We next conducted a multivariable binomial logistic analysis involving other potential risk factors determined before surgery and found that a low PLR was an independent risk factor for postoperative complications (odds ratio 3.32, 95%CI: 1.82-6.25, P < 0.001) along with male sex and total gastrectomy (Table 3). With respect to the types of complications, the low PLR group exhibited an increased prevalence of anastomotic leakage, leakage of pancreatic fluids, intraabdominal abscess and bowel obstruction compared to the high PLR group. However, the differences were not statistically significant (Figure 2). By contrast, the incidence of cardiopulmonary dysfunction was comparable between the two groups.

Subgroup analyses

To further evaluate the clinical effect of the preoperative PLR on the postoperative course, subgroup analyses were conducted according to age (< 65 years or ⩾ 65 years), preoperative BMI (< 22 or ⩾ 22),

Table 2 Comparison between two subgroups according to the preoperative platelet-lymphocyte ratio

Variables	PLR < 0.71 (*n* = 154)	PLR ⩾ 0.71 (*n* = 158)	*P* value
Age, median (range)	66 (20-86)	66 (34-96)	0.661
Sex			0.434
Male	112	121	
Female	42	37	
Diabetes mellitus			0.070
Absent	140	133	
Present	14	25	
Cardiac comorbidities			0.785
Absent	106	111	
Present	48	47	
Pulmonary comorbidities			0.021
Absent	149	143	
Present	5	15	
Preoperative symptoms			0.838
Absent	84	88	
Present	70	70	
Preoperative body mass index, mean ± SD	21.6 ± 3.3	22.2 ± 3.1	0.102
Tumor location			0.673
Entire	3	5	
Upper third	32	36	
Middle third	57	63	
Lower third	62	54	
Tumor size (mm)			0.043
< 50	78	98	
⩾ 50	76	60	
UICC cT factor			0.502
cT1	0	0	
cT2	69	81	
cT3	49	46	
cT4	36	31	
UICC cN factor			0.633
cN0	75	82	
cN1	51	42	
cN2	24	29	
cN3	4	5	
UICC clinical stage			0.823
Ⅰ B	45	53	
Ⅱ A	37	34	
Ⅱ B	32	37	
Ⅲ A	31	24	
Ⅲ B	7	7	
Ⅲ C	2	3	
Operative procedure			0.669
Total gastrectomy	52	57	
Distal gastrectomy	102	101	
Splenectomy			0.191
Absent	128	122	
Present	26	36	
Dissected lymph nodes, mean ± SD	37.6 ± 15.8	38.7 ± 18.0	0.823
Operative time (min), mean ± SD	233 ± 56	238 ± 74	0.720
Estimated blood loss (mL), median (range)	237 (1-2350)	245 (8-4267)	0.491
Postoperative complications			< 0.001
Absent	108	138	
Present	46	20	

PLR: Platelet-lymphocyte ratio; UICC: Union for International Cancer Control.

operative procedure (distal or total gastrectomy) and clinical disease stage. The low PLR group exhibited an increased incidence of postoperative complications for all subgroup analyses (Figure 3).

DISCUSSION

The PLR, a combination of circulating platelet and lymphocyte counts, is a representative index of systemic inflammation and immune status[9,17]. Accordingly, a decreased PLR value indicate both systemic inflammation and compromised immune reaction. Although preoperative systemic inflammation has been reported as an underlying characteristic that predisposes the host to postoperative infection, only limited evidence is available[18]. In this study, a significant relationship between the preoperative PLR and postoperative complications was demonstrated. The PLR exhibited the highest AUC value for the incidence of postoperative complications among the candidate parameters obtained from routine preoperative blood tests. A low preoperative PLR was identified as an independent risk factor for postoperative complications in the multivariable analysis. Moreover, a low preoperative PLR was associated with an increased incidence of postoperative complications independent of age, BMI, operative procedure and disease stage, indicating that the preoperative PLR is applicable to various clinical settings.

The PLR can be determined in every hospital and is the subject of interest as a marker of systemic inflammation in various clinical circumstances. Several reports indicate that the PLR can serve as a prognostic factor for digestive malignancies[9,18]. Accumulating evidence indicates that a systemic inflammatory response could play an important role in the development and progression of cancer. Inflammation is closely related to different stages of tumor development, including initiation, invasion, metastasis, immune surveillance and responses to therapy[7,8,10,19,20]. However, the clinical impact of the preoperative PLR on postoperative short-term outcomes, particularly in patients undergoing a gastrectomy, remains unclear. Our results suggested that surgeons can provide precise informed consent information and identify high-risk patients to tailor perioperative care as an attempt to ultimately improve postoperative short-term outcomes for GC patients undergoing a gastrectomy using this prediction tool. Recently, preoperative risk calculation systems based on large-scale integrated databases, such as the Surgical Risk Preoperative Assessment System (SURPAS) and the Physiologic and Operative Severity Score for the enUmeration of Mortality and morbidity (POSSUM) model, have been widely used in clinical practice[5,21]. Our data suggest that the PLR could at least become a potential constituent of integrated scoring systems for surgical risk assessment.

The association between a decreased PLR and the development of postoperative infectious complication

Table 3 Predictive factors for grade 2 or higher complications according to the Clavien-Dindo system: Univariate and multivariable analyses

Variables	Univariate			Multivariable		
	Odds ratio	95%CI	*P* value	Odds ratio	95%CI	*P* value
Age (≥ 65 yr)	1.26	0.73-2.23	0.410			
Gender (male)	4.22	1.88-11.3	< 0.001	4.17	1.78-11.5	< 0.001
Preoperative symptoms	0.95	0.55-1.64	0.864			
Diabetes mellitus	1.14	0.49-2.45	0.755			
Cardiac comorbidities	1.81	1.02-3.18	0.041	1.55	0.83-2.87	0.166
Pulmonary comorbidities	1.26	0.40-3.40	0.669			
Body mass index (≥ 22)	1.62	0.94-2.81	0.084			
Preoperative PLR (< 0.71)	2.94	1.66-5.35	< 0.001	3.32	1.82-6.25	< 0.001
Tumor location (lower third)	0.62	0.34-1.11	0.107			
Macroscopic tumor size (> 50 mm)	1.19	0.69-2.05	0.534			
UICC cT (cT4)	1.30	0.67-2.69	0.457			
UICC cN (cN1-3)	1.75	1.01-3.08	0.045	1.69	0.93-3.08	0.083
Operative procedure (total gastrectomy)	2.42	1.39-4.23	0.002	2.43	1.35-4.43	< 0.001

Analyses were performed using binomial logistic analysis. PLR: Platelet-lymphocyte ratio; UICC: Union for International Cancer Control.

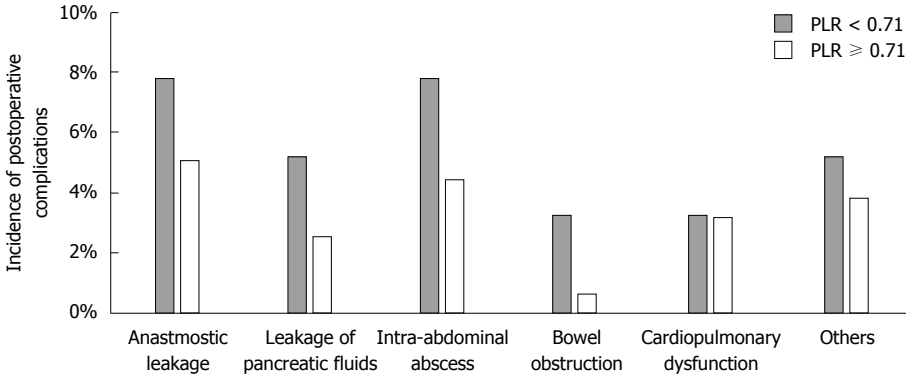

Figure 2 Comparison of the prevalence of each complication type between the high and low platelet-lymphocyte ratio groups.

is likely complex and remains unclear. One plausible explanation is that a decreased PLR may reflect four disadvantages for the postoperative course including an inflammatory status, immune disorders, malnutrition and a tendency for micro-vessel thrombosis[10,18]. A decreased PLR collectively reflects a reduced TLC (compromised cell-mediated immunity and malnutrition) and an increased platelet count (inflammation and high thromphophilic diathesis). Lymphocytopenia is associated with malnutrition and cellular immunosuppression[22-24]. A pro-inflammatory status leads to compromised cell-mediated immunity and an impaired T-lymphocytic response *via* cytokines[25]. The ability of myeloid cells to synthesize pro-inflammatory and anti-inflammatory mediators is affected by their previous state[10,18]. Malnutrition is a major cause of delayed wound healing[11,15]. A decreased lymphocyte-mediated antibacterial immune reaction may weaken the lymphocyte-mediated antibacterial cellular immune response and contribute to increasing bacterial invasion and growth[10,18]. The platelet count is recognized as a marker of a systemic inflammatory response and potential micro-vessel thrombosis[7,9,17]. Formation of micro-vessel thrombosis inhibits wound healing via the deterioration of blood circulation in tissues. Eventually, the crosstalk of these complex factors increases the incidence of postoperative complications. In addition, our findings raised two questions. First, why is the PLR was more sensitive than the other indices, such as the PNI and GPS? We hypothesize that the PLR is indicative of four elements, including inflammatory status, immune disorders, malnutrition and a tendency for micro-vessel thrombosis, and therefore it was more closely associated with postoperative complications than other indices. Another question is whether preoperative modification of the PLR by anti-inflammatory treatment and nutritional support reduces the adverse effects on the postoperative clinical course. Further investigations are needed to answer these questions.

The current study also had several limitations that should not be ignored. This was a retrospective study of a limited number of patients from a single institute. Thus, our findings require validation by further large-scale prospective studies. To further evaluate the association of the PLR with the host inflammatory and nutritional status, information on levels of cytokines and rapid turnover proteins is required.

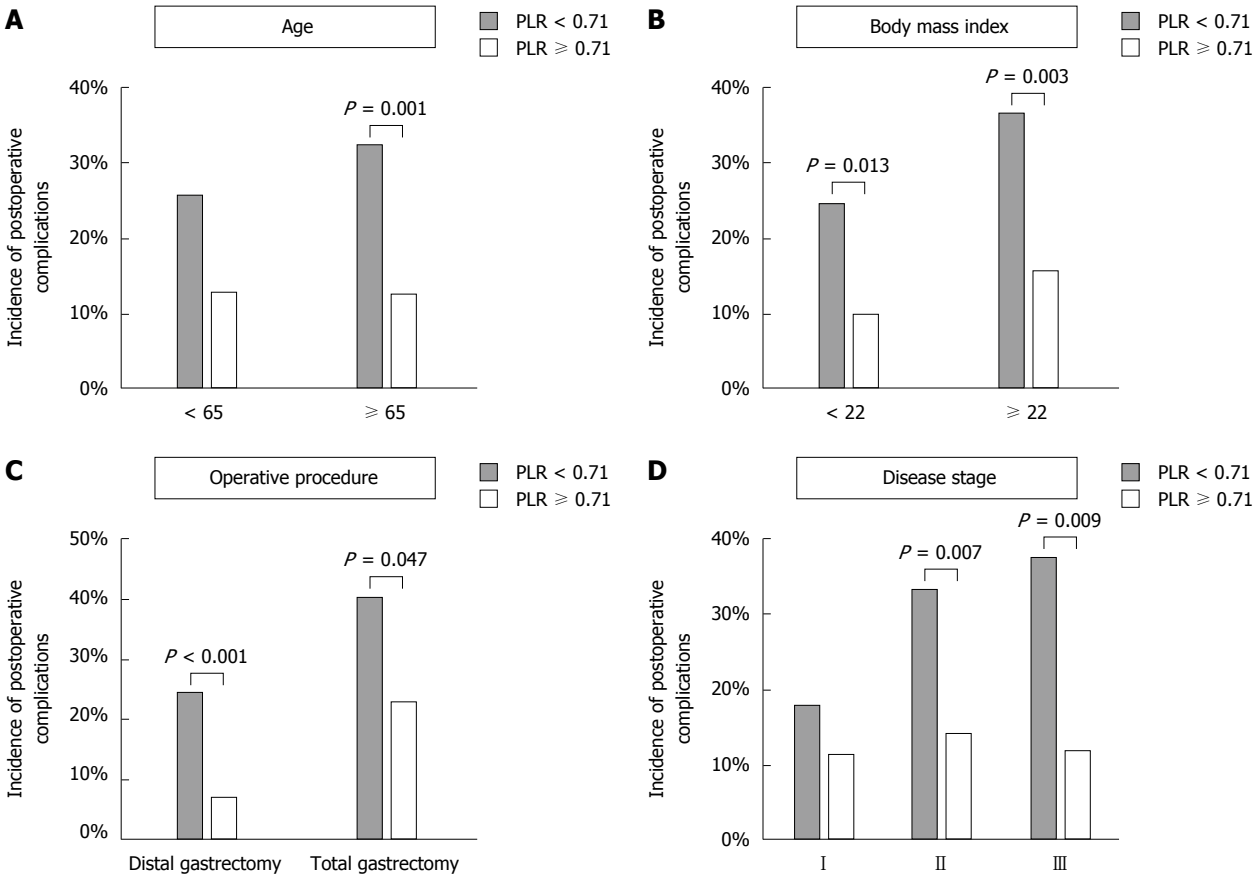

Figure 3 Subgroup analyses. The morbidity rates were compared between the high and low PLR groups according to age (A), body mass index (B), operative procedure (C) and clinical disease stage (D). Comparison of predictive value of potential indicators. PLR: Platelet-lymphocyte ratio.

Taken together, the results indicate that the preoperative PLR is a simple and useful predictor of postoperative complications in patients who undergo a gastrectomy with systemic lymphadenectomy for GC. In the future, the development of an integrated risk stratification system using the PLR may facilitate physicians' decision-making and contribute to the informed consent process before a gastrectomy.

COMMENTS

Background

Patients undergoing a gastrectomy with systemic lymphadenectomy for gastric cancer occasionally experience clinically relevant postoperative complications. Development of a prediction tool based on preoperatively determined factors would be helpful to identify individuals at risk of postoperative complications for whom precise informed consent and tailored perioperative management could be provided.

Research frontiers

Platelet-lymphocyte ratio (PLR) showed the highest predictive value for postoperative complications among the 21 parameters. It was found to be an independent risk factor in a multivariate binomial logistic analysis.

Innovations and breakthroughs

The authors found that PLR served as a predictor of postoperative complications in all subgroups classified according to age, body mass index, operative procedure and clinical disease stage.

Applications

PLR is a simple and useful predictor of postoperative complications in patients who undergo a gastrectomy with systemic lymphadenectomy for gastric cancer. It may facilitate physicians' decision-making and contribute to the informed consent process before a gastrectomy.

Terminology

PLR is a platelet-lymphocyte ratio (total lymphocyte count/platelet count × 100) and is a representative index of systemic inflammation and immune status.

Peer-review

This is an interesting, single center retrospective study on clinical utility of preoperative predictors of postoperative complications for clinical T2-4 gastric cancer. The authors made a high quality statistical analysis and found a new statistical, easy, reproducible and overall available index, the PLR, to predict the postoperative complications after radical gastrectomy for clinical T2-4 gastric cancer.

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ORIGINAL ARTICLE

Case Control Study

Colors of vegetables and fruits and the risks of colorectal cancer

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Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at shinaesun@snu.ac.kr and jskim@ncc.re.kr.

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Abstract

AIM

To investigate the relationship between the colors of vegetables and fruits and the risk of colorectal cancer in Korea.

METHODS

A case-control study was conducted with 923 colorectal cancer patients and 1846 controls recruited from the National Cancer Center in Korea. We classified vegetables and fruits into four groups according to the color of their edible parts (*e.g.*, green, orange/yellow, red/purple and white). Vegetable and fruit intake level was classified by sex-specific tertile of the control group. Logistic regression models were used for estimating the odds ratios (OR) and their 95% confidence intervals (CI).

RESULTS

High total intake of vegetables and fruits was strongly associated with a reduced risk of colorectal cancer in women (OR = 0.32, 95%CI: 0.21-0.48 for highest *vs* lowest tertile) and a similar inverse association was observed for men (OR = 0.60, 95%CI: 0.45-0.79). In the analysis of color groups, adjusted ORs (95%CI) comparing the highest to the lowest of the vegetables and fruits intake were 0.49 (0.36-0.65) for green, and 0.47 (0.35-0.63) for white vegetables and fruits in men. An inverse association was also found in women for green, red/purple and white vegetables and fruits. However, in men, orange/yellow vegetables and fruits (citrus fruits, carrot, pumpkin, peach, persimmon, ginger) intake was linked to an increased risk of colorectal cancer (OR = 1.61, 95%CI: 1.22-2.12).

CONCLUSION

Vegetables and fruits intake from various color groups may protect against colorectal cancer.

Key words: Vegetable and fruits; Colorectal cancer; Korea

Core tip: Although many studies have focused on the associations between vegetable and fruit intake and health, few studies have classified vegetables and fruits by their colors, which reflect their unique contents of phytochemicals and micronutrients. In the current study, most color groups of vegetables and fruits showed protective benefits against colorectal cancer regardless of the anatomical subsites.

Lee J, Shin A, Oh JH, Kim J. Colors of vegetables and fruits and the risks of colorectal cancer. *World J Gastroenterol* 2017; 23(14): 2527-2538 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i14/2527.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i14.2527

INTRODUCTION

Vegetables and fruits contain nutrients such as vitamins, minerals, folate, dietary fiber, plant sterols, carotenoids and various phytochemicals[1,2] . These nutrients may reduce mortality and prevent chronic diseases, including various cancers, cardiovascular diseases and even mental illnesses, through their antitumor activity as well as their anti-obesity, anti-oxidant and anti-inflammatory agents[1,3-7].

According to the latest research on the prevention of colorectal cancer from the Continuous update project of the World Cancer Research Fund/American Institute for Cancer Research (WCRF/AICR), which was published in 2011, non-starchy vegetables and fruits have been evaluated as a "limited-suggestive" preventive factor[8]. A recent meta-analysis of 15 cohort studies of vegetable and fruit intake and the risk of colorectal cancer found that there was a small (8%) reduction and a nonlinear inverse association between colorectal cancer risk and the intake of vegetables and fruits[9]. The relationship between cruciferous vegetables, citrus fruits, brassica vegetables, leafy vegetables, root vegetables and total vegetables and fruits consumption were not consistent[10-15].

Phytochemicals from vegetables and fruits contain many colorful and dark pigments, such as flavonoid and polyphenols, and may be distinguished (in terms of their various physiological effects and actions) by their specific colors. A previous study suggested classifying vegetables and fruits according to their nutritional phytochemicals when providing guidelines for the public[16]. Pennington and Fish have classified 9 color groupings of vegetable and fruit subgroups based on a consideration their unique nutritional values, features and potential correlations[17,18].

There have been studies of vegetable and fruit classification, by color, for stroke[19], coronary heart disease[20], and colorectal cancer[15]. Our present case-control study, therefore, explored the association between vegetables and fruits color groups and colorectal cancer risk in the Korean population.

MATERIALS AND METHODS

Study subjects

The colorectal cancer cases were recruited from the Center for Colorectal Cancer of the National Cancer Center in Korea between August 2010 and August 2013. Among 1427 eligible patients, 1070 agreed to participate in the study. Colorectal cancer cases with incomplete semi-quantitative food frequency questionnaire (SQFFQ) data (145 cases) and those with implausible energy intakes below 500 Kcal/d or above 4000 Kcal/d (2 cases) were excluded. Controls were persons who received health screenings provided by the National Health Insurance Corporation between October 2007 and December 2014 at the same institute. Among 14201 potential control participants, individuals with incomplete SQFFQ (n = 5044) and with implausible energy intakes (n = 120) were excluded. Patients and eligible controls were matched in a 1:2 ratio according to their sex and 5 year age groups. Ultimately, there were 923 cases and 1846 controls whose data were used in the final analysis. All the participants provided written informed consent, and this study's protocol was approved by the Institutional review board of the National Cancer Center (IRB No. NCCNCS-10-350 and No. NCC 2015-0202).

Data collection

A trained dietitian performed questionnaire surveys through face-to-face interviews. Information on

Table 1 Classification of vegetables and fruits according to type and color group[1]

Color group	Vegetables and fruits type	Vegetables and fruits item
Green (23.2%)[2]	Dark green leafy vegetables (27.1%)[3]	Water dropwort, mugwort, crown daisy, spinach, perilla leaf, chicory, kale, pumpkin leaf, leak beet
	Lettuces (10.6%)	Lettuce
	Other green fruits and vegetables (62.3%)	Melon, zucchini, green cucumber, green pepper, cabbages, broccoli, celery
Orange/yellow (17.5%)	Citrus fruits (57.5%)	Citrus fruits juices, orange, mandarin orange, kumquat
	Other orange/yellow fruits and vegetables (42.5%)	Carrot, pumpkin, peach, persimmon, ginger
Red/purple (19.0%)	Berries (38.3%)	Strawberry , grape
	Other red fruits and vegetables (61.7%)	Watermelon, tomato, red cabbage, red pepper, plum
White (40.3%)	Allium family bulbs (15.1%)	Garlic, leek, onion
	Hard fruits (41.8%)	Apple, pear
	Cauliflower (13.4%)	Asian radish
	Other white fruits and vegetables (29.7%)	Oriental melon, mushroom, banana, deodeok, burdock, lotus root, balloon flower root

[1]Vegetables and fruits were classified into subgroup as proposed by Pennington and Fisher; [2]Proportion of color group to total vegetables and fruits; [3]Proportion of vegetables and fruits type to vegetables and fruits by color group.

general characteristics, family history of cancer, alcohol consumption, cigarette smoking, and exercise habits was obtained using a structured questionnaires. Dietary information was assessed using the semi-quantitative food frequency questionnaire (SQFFQ) developed by the Korea Centers for Disease Control and Prevention[21]. The SQFFQ was designed to measure typical food intake habits during the course of one year. The reliability and validity of this questionnaire have been previously reported[21]. Subjects were queried by a trained dietitian on their usual intake amount of 106 food items during the last 12 mo before the interview. Daily vegetable and fruit intake and calorie intake were calculated using the Nutritional Analysis Program for Professionals ver. 4.0 (CAN-Pro 4.0 the Korean Nutrition Society, 2012, Seoul, Korea). Vegetables and fruits were classified into 4 color groups according to Pennington and Fish's[17,18] categories (*e.g.*, green, orange/yellow, red/purple and white) (Table 1). On the basis of outcomes form the Food Balance Wheels (Ministry of Health and Welfare, Dietary Reference Intakes for Koreans, 2015), potatoes and sweet potatoes, which have high starch content, were not included as vegetables. Additionally, we did not include kimchi, pickled vegetables and jam as vegetables and fruits, because of their high salt and sugar content. And the fruit juice beverages were included in the analysis. We have performed an analysis according to the anatomical location of the origin of cancer: proximal colon (cecum, ascending colon, hepatic flexure, transverse colon, splenic flexure); distal colon (descending colon, sigmoid-descending colon junction, sigmoid colon); and rectum (rectosigmoid colon, rectum).

Statistical analysis

Chi-square tests were used to compare the distribution of general characteristics and health related behavior factors among cases and controls. Intake levels of vegetables and fruits were categorized into sex-specific tertiles according to the distribution among control groups. The potential confounding variable considered were age, education, alcohol consumption, regular exercise, body mass index (BMI), fiber intake, red meat consumption, processed meat consumption, and energy intake, all of which were selected based on the literature[9,13,22-24]. After considering multi-collinearity, we finally adjusted age, education level, alcohol consumption, BMI, regular exercise, red meat consumption, processed meat consumption, and total energy intake by residual methods. Nutrient intakes were adjusted for total individual energy intakes using the residual method[25]. Binary and polytomous logistic regression models were used to assess the ORs and their 95%CIs for the association between the colors of the vegetables and fruits consumed and the risk of colorectal cancer. All statistical analyses were performed using SAS software (version 9.4; SAS Institute Inc. Cary, NC, United States).

RESULTS

The characteristics of the study subjects are presented in Table 2. Male colorectal cancer patients showed differences compared to controls in marital status, education level, household income, obesity, smoking status, alcohol consumption and regular exercise. The female subjects showed a similar pattern, but the cancer patients had a higher percentage of obese individuals and current smokers.

Table 3 presents consumption of vegetables and fruits for the cases and controls, separated by sex. Total energy intake was higher among controls in both sexes; thus, the energy adjusted average intake levels of vegetables and fruits were compared. Among cancer cases, consumption of total vegetables and fruits, vegetables, fruits, color group vegetables and fruits and even red meat was lower than controls.

Table 4 shows the ORs and the 95%CIs for the colors of the vegetables and fruits consumed and the

Table 2 General characteristics of the study subjects *n* (%)

Variable	Male (*n* = 1875)		*P* value[1]	Female (*n* = 894)		*P* value[1]
	Case (*n* = 625)	Control (*n* = 1250)		Case (*n* = 298)	Control (*n* = 596)	
Age group (yr)			0.997			0.994
-49	128 (20.5)	258 (20.6)		82 (27.5)	166 (27.9)	
50-59	227 (36.3)	453 (36.2)		111 (37.3)	221 (37.1)	
60+	270 (43.2)	539 (43.1)		105 (35.2)	209 (35.1)	
Missing	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	
Marital status			< 0.001			< 0.001
Married	557 (89.1)	1162 (93.0)		216 (73.0)	493 (83.4)	
Single	66 (10.6)	72 (5.8)		80 (27.0)	98 (16.6)	
Missing	2 (0.3)	16 (1.3)		2 (0.7)	5 (0.8)	
Education level			< 0.001			< 0.001
Under middle school	183 (29.3)	175 (14.0)		138 (46.3)	106 (18.1)	
High school	266 (42.6)	329 (26.3)		103 (34.6)	258 (44.0)	
College or more	176 (28.2)	712 (57.0)		57 (19.1)	223 (38.0)	
Missing	0 (0.0)	34 (2.7)		0 (0.0)	9 (1.5)	
Income (10000won/mo)			< 0.001			< 0.001
< 200	222 (35.5)	254 (20.3)		99 (33.2)	134 (25.0)	
200-400	253 (40.5)	534 (42.7)		134 (45.0)	218 (40.7)	
> 400	150 (24.0)	363 (29.0)		65 (21.8)	184 (34.3)	
Missing	0 (0.0)	99 (7.9)		0 (0.0)	60 (10.1)	
Body mass index (kg/m^2)			< 0.001			0.270
< 25	432 (69.1)	734 (58.7)		207 (69.5)	435 (73.0)	
⩾ 25	192 (30.7)	516 (41.3)		91 (30.5)	161 (27.0)	
Missing	1 (0.2)	0 (0.0)		0 (0.0)	0 (0.0)	
Smoking status			0.076			< 0.001
Non-smoker	145 (23.2)	245 (19.6)		264 (88.6)	571 (95.8)	
Ex-smoker	303 (48.5)	671 (53.7)		15 (5.0)	16 (2.7)	
Current smoker	177 (28.3)	334 (26.7)		19 (6.4)	9 (1.5)	
Missing	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	
Alcohol consumption			< 0.001			0.186
Non-drinker	107 (17.1)	199 (15.9)		172 (57.7)	362 (60.7)	
Ex-drinker	103 (16.5)	136 (10.9)		26 (8.7)	33 (5.5)	
Current drinker	415 (66.4)	915 (73.2)		100 (33.6)	201 (33.7)	
Missing	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	
Regular exercise			< 0.001			< 0.001
No	387 (61.9)	490 (39.2)		225 (75.5)	262 (44.0)	
Yes	238 (38.1)	715 (57.2)		73 (24.5)	333 (56.0)	
Missing	0 (0.0)	45 (3.6)		0 (0.0)	1 (0.2)	
Family history of cancer			0.002			0.141
No	392 (62.7)	686 (54.9)		171 (57.4)	311 (52.2)	
Yes	233 (37.3)	560 (44.8)		127 (42.6)	285 (47.8)	
Missing	0 (0.0)	4 (0.3)		0 (0.0)	0 (0.0)	
Family history of colorectal cancer			< 0.001			0.926
No	560 (89.6)	1188 (95.0)		277 (93.0)	555 (93.1)	
Yes	65 (10.4)	58 (4.6)		21 (7.1)	41 (6.9)	
Missing	0 (0.0)	4 (0.3)		0 (0.0)	0 (0.0)	

[1]*P* values were calculated by χ^2 test.

risks of colorectal cancer. After adjustments for the confounding variables, we found that higher intake of total vegetables and fruits (OR = 0.60, 95%CI: 0.45-0.79, highest *vs* lowest tertiles); vegetables (OR = 0.48, 95%CI: 0.36-0.64); green vegetables and fruits (OR = 0.49, 95%CI: 0.36-0.65); and white vegetables and fruits (OR = 0.47, 95%CI: 0.35-0.63) reduced the risks of colorectal cancer for men. However, for orange/yellow vegetables and fruits, a significant association with the risks of colorectal cancer was found (OR = 1.61, 95%CI: 1.22-2.12). In women, all categories of vegetables and fruits intake showed decreased risk of colorectal cancer (OR = 0.32, 95%CI: 0.21-0.48 for total vegetables and fruits; OR = 0.37, 95%CI: 0.24-0.57 for vegetables; OR = 0.41, 95%CI: 0.27-0.63 for fruits; OR = 0.25, 95%CI: 0.16-0.40 for green vegetables and fruits; OR = 0.66, 95%CI: 0.44-0.99 for red/purple vegetables and fruits; OR = 0.34, 95%CI: 0.22-0.52 for white vegetables and fruits).

In the analysis of orange/yellow vegetables and fruits separately, orange/yellow fruits intake reduced the risks of colorectal cancer in women (OR = 0.64, 95%CI: 0.43-0.97). We found that higher intake of orange/yellow vegetables elevated the risks of colorectal cancer in both sexes (OR = 2.41, 95%CI:

Table 3 Intake of vegetables and fruits between cases and controls

Total energy adjusted intake (g/d), mean ± SD	Male (n = 1875)		Female (n = 894)	
	Case (n = 625)	Control (n = 1250)	Case (n = 298)	Control (n = 596)
Total energy intake (kcal/d)	2127.7 ± 509.1	1731.6 ± 545.8	1814.4 ± 523.5	1604.6 ± 577.4
Total vegetables and fruits	279.4 ± 155.7	350.2 ± 236.7	343.1 ± 192.8	470.7 ± 383.4
Total vegetables	148.5 ± 77.3	186.7 ± 126.2	155.8 ± 85.4	205.3 ± 138.2
Total fruits	125.0 ± 115.3	174.5 ± 197.8	185.9 ± 152.1	271.7 ± 230.0
Green vegetables and fruits	64.8 ± 38.0	86.2 ± 69.6	72.7 ± 52.1	105.5 ± 84.5
Orange/yellow vegetables and fruits	49.4 ± 45.4	54.2 ± 66.8	75.4 ± 71.2	95.2 ± 97.7
Orange/yellow vegetable	10.1 ± 12.1	9.3 ± 17.0	13.0 ± 16.3	11.7 ± 23.1
Orange/yellow fruits	37.7 ± 46.2	57.0 ± 109.1	65.0 ± 84.7	94.7 ± 117.0
White vegetables and fruits	105.9 ± 65.4	149.8 ± 120.4	128.8 ± 100.3	186.2 ± 135.0
Red/purple vegetables and fruits	55.2 ± 65.9	66.1 ± 88.8	66.0 ± 62.6	89.1 ± 89.8
Red meat	56.0 ± 36.2	64.4 ± 41.9	40.9 ± 26.9	43.7 ± 28.7
Processed meat	0.5 ± 1.8	3.5 ± 25.1	1.9 ± 13.8	1.7 ± 6.1

Mean of vegetables and fruits intake were adjusted for the total individual energy intakes using the residual method.

1.83-3.16 for men; OR = 2.28, 95%CI: 1.55-3.34 for women). In the subsite analysis (Table 5), similar associations by subsite were observed for both men and women.

DISCUSSION

In this case control study, we investigated the relationship between vegetables and fruits groups categorized by color and the risks of colorectal cancer. The investigation revealed that the green vegetables and fruits and white vegetables and fruits color groups and total vegetables and fruits intake were strongly related to a reduced risk of colorectal cancer in men and women. In addition, it was shown that in women, the total amount of fruit consumed, as well as consumption of the red/purple color groups, attenuated colorectal cancer risk. However, no significant association was found for the red/purple color groups in men. Surprisingly, a high intake from the orange/yellow vegetables and fruits color group was associated with a higher risk of colorectal cancer in men.

The protective effect of total vegetables and fruits intake as related to colorectal cancer risk was consistent with previous case-control studies[12,26-28] and meta-analysis[9]. However, recent cohort studies[13,14,23,29], and a recent case- control study[12], do not comply with our results.

In the present study, green vegetables and fruits intake was shown to be inversely associated with the risk of CRC in both sexes. Green vegetables and fruits are thought to decrease the risk of CRC through their high folate, fiber, lutein, sulforaphane and indole level, which induce apoptosis in cancer cells and inhibit cell damage and the growth of cancer cells[30,31]. The Netherlands Cohort Study[10], as well as a case-control study for Guangzhou (in men)[15] and the NIH-AARP study[11] reported the beneficial effects of green vegetables and fruits. However, other cohort studies and case control studies have produced null findings[12,13,32,33].

This study suggested that high white vegetables and fruits intake has protective effects on colorectal cancer risk. White vegetables and fruits contains various phytochemicals and nutrients, such as the polysaccharides of apples, theglucans of mushroom, saponins of root and bulb vegetables, and the quercetin of onions and apples, which play important roles in antioxidant activity, reduction of DNA damage, and anticancer activity[34]. However, epidemiological studies of white vegetables and fruits intake are still contradictory. In the case of apples, with the exception of one study[33], most research has shown a beneficial significant association[12,35] or no association between apple intake and colorectal cancer risk. Several recent meta-analyses have been published[36-39] on bulbs in the allium family, and the results of these papers show that garlic consumption is not associated with colorectal cancer.

Studies that classify vegetables and fruits by color are rare regardless of the disease. One case-control study was conducted in China[15]. In a case-control study from Switzerland, citrus fruit, a main component of the orange/yellow vegetables and fruits category, was found to be significantly inversely associated with colorectal cancer risk[40]. However, most studies show no significant associations with orange/yellow vegetables and fruits consumption[10,12,13,29,32,33]. In two case control studies conducted in China and Hawaii, it was found that high orange/yellow vegetables and fruits intake reduces colorectal cancer risk[15,41]. Orange/yellow vegetables and fruits are known to be rich in carotene, which can function as provitamin A[42]. Vitamin A may have a positive effect by controlling the growth and metastasis of cancer cells and may act as an antioxidant in reducing cancer[43]. However, our study found that high orange/yellow vegetable and fruit intake was significantly associated with increased risk of colorectal cancer for men. The Nurse' Health Study and Health Professionals Follow-Up Study conducted in the United States suggested that citrus

Table 4 Odds ratios and 95% confidence intervals for colorectal cancer risk in relation to intake of vegetables and fruits

Total energy adjusted vegetables and fruits intake	Male (*n* = 1875)				Female (*n* = 894)			Total (*n* = 2769)		
	Controls /cases (*n*)	Age-adjusted OR (95%CI)	Multivariate OR[1] (95%CI)		Controls /cases (*n*)	Age-adjusted OR (95%CI)	Multivariate OR[1] (95%CI)	Controls /cases (*n*)	Age-adjusted OR (95%CI)	Multivariate OR[1] (95%CI)
Total vegetables and fruits (g/d)										
T1 (< 224.2)	417/275	1.00	1.00	T1 (< 317.7)	199/166	1.00	1.00	616/441	1.00	1.00
T2 (224.2-< 380.0)	416/230	0.83 (0.67-1.04)	0.94 (0.74-1.21)	T2 (317.7- < 534.8)	199/91	0.55 (0.39-0.75)	0.60 (0.42-0.85)	615//321	0.72 (0.60-0.87)	0.81 (0.67-0.99)
T3 (≥ 380.0)	417/120	0.43 (0.34-0.56)	0.60 (0.45-0.79)	T3 (≥ 534.8)	198/41	0.25 (0.17-0.37)	0.32 (0.21-0.48)	615/161	0.36 (0.29-0.45)	0.50 (0.40-0.63)
P for trend[2]		< 0.001	< 0.001			< 0.001	< 0.001		< 0.001	< 0.001
Total vegetables (g/d)										
T1 (< 123.0)	416/256	1.00	1.00	T1 (< 135.9)	198/143	1.00	1.00	614/399	1.00	1.00
T2 (123.0-< 203.6)	418/270	1.05 (0.84-1.30)	1.19 (0.93-1.52)	T2 (135.9- < 219.1)	200/110	0.76 (0.55-1.04)	0.89 (0.62-1.26)	618/380	0.94 (0.79-1.13)	1.05 (0.86-1.28)
T3 (≥ 203.6)	416/99	0.38 (0.29-0.50)	0.48 (0.36-0.64)	T3 (≥ 219.1)	198/45	0.31 (0.21-0.46)	0.37 (0.24-0.57)	614/114	0.36 (0.29-0.45)	0.43 (0.34-0.55)
P for trend		< 0.001	< 0.001			< 0.001	< 0.001		< 0.001	< 0.001
Total fruits (g/d)										
T1 (< 68.3)	416/224	1.00	1.00	T1 (< 135.0)	198/129	1.00	1.00	614/353	1.00	1.00
T2 (68.3-< 178.1)	418/265	1.18 (0.94-1.47)	1.36 (1.06-1.74)	T2 (135.0- < 307.1)	199/124	0.96 (0.70-1.31)	1.03 (0.73-1.46)	617/389	1.09 (0.91-1.31)	1.21 (1.00-1.48)
T3 (≥ 178.1)	416/136	0.61 (0.47-0.78)	0.77 (0.58-1.02)	T3 (≥ 307.1)	199/45	0.35 (0.23-0.51)	0.41 (0.27-0.63)	615/181	0.51 (0.41-0.63)	0.67 (0.53-0.84)
P for trend		< 0.001	0.017			< 0.001	< 0.001		< 0.001	< 0.001
Green vegetables and fruits (g/d)										
T1 (< 48.8)	417/238	1.00	1.00	T1 (< 61.0)	199/151	1.00	1.00	616/389	1.00	1.00
T2 (48.8-< 93.6)	417/280	1.17 (0.94-1.46)	1.21 (0.94-1.54)	T2 (61.0-< 114.2)	198/115	0.76 (0.56-1.04)	0.89 (0.63-1.27)	615/395	1.01 (0.84-1.21)	1.06 (0.87-1.30)
T3 (≥ 93.6)	416/107	0.45 (0.34-0.58)	0.49 (0.36-0.65)	T3 (≥ 114.2)	199/32	0.21 (0.14-0.32)	0.25 (0.16-0.40)	615/139	0.35 (0.28-0.44)	0.39 (0.31-0.50)
P for trend		< 0.001	< 0.001			< 0.001	< 0.001		< 0.001	< 0.001
Red/purple vegetables and fruits (g/d)										
T1 (< 22.1)	416/191	1.00	1.00	T1 (< 39.5)	199/112	1.00	1.00	615/303	1.00	1.00
T2 (22.1- < 62.2)	417/266	1.39 (1.10-1.75)	1.63 (1.26-2.11)	T2 (39.5-< 98.5)	199/121	1.08 (0.78-1.49)	1.21 (0.84-1.73)	616/387	1.27 (1.06-1.54)	1.46 (1.19-1.79)
T3 (≥ 62.2)	417/168	0.88 (0.68-1.12)	1.10 (0.83-1.44)	T3 (≥ 98.5)	198/65	0.58 (0.41-0.84)	0.66 (0.44-0.99)	615/233	0.77 (0.63-0.94)	0.96 (0.77-1.20)
P for trend		0.042	0.706			0.002	0.027		< 0.001	0.033
Orange /yellow vegetables and fruits (g/d)										
T1 (< 21.1)	416/162	1.00	1.00	T1 (< 40.7)	199/89	1.00	1.00	615/251	1.00	1.00
T2 (21.1- < 54.0)	417/264	1.63 (1.28-2.07)	1.91 (1.47-2.49)	T2 (40.7- < 100.6)	198/140	1.58 (1.14-2.20)	1.77 (1.23-2.56)	615/404	1.61 (1.33-1.95)	1.78 (1.45-2.20)
T3 (≥ 54.0)	417/199	1.23 (0.96-1.58)	1.61 (1.22-2.12)	T3 (≥ 100.6)	199/69	0.77 (0.53-1.12)	0.85 (0.56-1.27)	616/268	1.07 (0.87-1.31)	1.33 (1.06-1.66)
P for trend		0.618	0.021			0.051	0.163		0.286	0.576
Orange /yellow fruits (g/d)										
T1 (< 15.9)	416/230	1.00	1.00	T1 (< 32.5)	199/105	1.00	1.00	615/335	1.00	1.00
T2 (15.9- < 47.9)	417/230	1.00 (0.79-1.25)	1.17 (0.91-1.51)	T2 (32.5- < 90.6)	198/130	1.24 (0.90-1.72)	1.43 (1.00-2.06)	616/360	1.07 (0.89-1.29)	1.20 (0.98-1.47)
T3 (≥ 47.9)	417/165	0.72 (0.56-0.91)	0.98 (0.75-1.28)	T3 (≥ 90.6)	199/63	0.60 (0.42-0.87)	0.64 (0.43-0.97)	615/228	0.68 (0.56-0.83)	0.85 (0.69-1.06)
P for trend		0.003	0.003			0.002	0.002		< 0.001	< 0.001
Orange /yellow vegetable (g/d)										
T1 (< 2.7)	416/144	1.00	1.00	T1 (< 3.2)	198/77	1.00	1.00	614/221	1.00	1.00
T2 (2.7- < 7.7)	417/188	1.30 (1.01-1.68)	1.47 (1.11-1.95)	T2 (3.2- < 9.1)	200/69	0.89 (0.61-1.30)	1.14 (0.75-1.73)	617/257	1.16 (0.94-1.43)	1.30 (1.04-1.64)
T3 (≥ 7.7)	417/293	2.09 (1.63-2.67)	2.41 (1.83-3.16)	T3 (≥ 9.1)	198/152	2.01 (1.43-2.83)	2.28 (1.55-3.34)	615/445	2.06 (1.69-2.51)	2.19 (1.77-2.73)
P for trend		< 0.001	< 0.001			< 0.001	< 0.001		< 0.001	< 0.001

White vegetables and fruits (g/d)										
T1 (< 87.9)	416/301	1	1	T1 (< 109.9)	199/156	1	1	615/457	1	1
T2 (87.9- < 153.9)	418/219	0.72 (0.57-0.90)	0.84 (0.66-1.08)	T2 (109.9- < 198.0)	199/99	0.63 (0.46-0.87)	0.78 (0.55-1.11)	617/318	0.69 (0.58-0.83)	0.8 (0.66-0.98)
T3 (≥ 153.9)	416/105	0.34 (0.27-0.45)	0.47 (0.35-0.63)	T3 (≥ 198.0)	198/43	0.28 (0.19-0.41)	0.34 (0.22-0.52)	614/148	0.32 (0.26-0.40)	0.43 (0.34-0.55)
P for trend		< 0.001	< 0.001			< 0.001	< 0.001		< 0.001	< 0.001

[1]Adjusted by age, education, alcohol consumption, BMI, regular exercise, red meat, processed meat and total energy intake; [2]Test for trend calculated with the median intake for each category of vegetables and fruits as a continuous variable.

Table 5 Odds ratios and 95% confidence intervals for colorectal cancer subsites in relation to intake of vegetables and fruits

	Control		Proximal colon		No.	Distal colon		No.	Rectum	
	No.	No.	Age-adjusted OR (95%CI)	Multivariate OR[1] (95%CI)		Age-adjusted OR (95%CI)	Multivariate OR[1] (95%CI)		Age-adjusted OR (95%CI)	Multivariate OR[1] (95%CI)
Men										
Total vegetables and fruits (g/d)										
T1 (< 224.2)	417	46	1.00	1.00	76	1.00	1.00	145	1.00	1.00
T2 (224.2- < 380.0)	416	45	0.97 (0.63-1.49)	1.08 (0.68-1.69)	73	0.95 (0.67-1.35)	1.10 (0.76-1.58)	107	0.74 (0.55-0.98)	0.82 (0.61-1.12)
T3 (≥ 380.0)	417	22	0.47 (0.28-0.80)	0.63 (0.36-1.09)	29	0.38 (0.24-0.59)	0.50 (0.32-0.80)	68	0.47 (0.34-0.64)	0.67 (0.47-0.94)
P for trend[2]			0.004	0.086		< 0.001	0.003		< 0.001	0.018
Total vegetables (g/d)										
T1 (< 123.0)	416	40	1.00	1.00	66	1.00	1.00	142	1.00	1.00
T2 (123.0-< 203.6)	418	46	1.13 (0.72-1.77)	1.26 (0.79-2.01)	88	1.32 (0.93-1.87)	1.50 (1.04-2.16)	130	0.91 (0.69-1.20)	1.05 (0.77-1.41)
T3 (≥ 203.6)	416	27	0.67 (0.40-1.11)	0.80 (0.47-1.37)	24	0.36 (0.22-0.59)	0.44 (0.27-0.74)	48	0.34 (0.24-0.48)	0.43 (0.29-0.63)
P for trend			0.089	0.34		< 0.001	0.002		< 0.001	< 0.001
Total fruits (g/d)										
T1 (< 68.3)	416	37	1.00	1.00	69	1.00	1.00	113	1.00	1.00
T2 (68.3- < 178.1)	418	52	1.38 (0.89-2.16)	1.59 (1.01-2.52)	76	1.09 (0.76-1.55)	1.26 (0.88-1.83)	129	1.14 (0.85-1.52)	1.30 (0.96-1.78)
T3 (≥ 178.1)	416	24	0.64 (0.38-1.09)	0.83 (0.47-1.44)	33	0.47 (0.31-0.74)	0.59 (0.38-0.94)	78	0.69 (0.50-0.95)	0.90 (0.63-1.27)
P for trend			0.035	0.269		< 0.001	0.01		0.008	0.318
Green vegetables and fruits (g/d)										
T1 (< 48.8)	417	39	1.00	1.00	64	1.00	1.00	129	1.00	1.00
T2 (48.8- < 93.6)	417	49	1.24 (0.80-1.94)	1.26 (0.79-1.99)	87	1.35 (0.95-1.92)	1.43 (0.99-2.07)	136	1.05 (0.80-1.39)	1.08 (0.79-1.46)
T3 (≥ 93.6)	416	25	0.63 (0.38-1.07)	0.67 (0.39-1.16)	27	0.42 (0.26-0.67)	0.46 (0.28-0.74)	55	0.43 (0.30-0.60)	0.47 (0.32-0.68)
P for trend			0.048	0.092		< 0.001	< 0.001		< 0.001	< 0.001
Red/purple vegetables and fruits (g/d)										
T1 (< 22.1)	416	34	1.00	1.00	60	1.00	1.00	91	1.00	1.00
T2 (22.1- < 62.2)	417	48	1.40 (0.88-2.22)	1.63 (1.01-2.04)	73	1.21 (0.84-1.75)	1.39 (0.95-2.04)	138	1.52 (1.13-2.04)	1.84 (1.33-2.53)
T3 (≥ 62.2)	417	31	0.90 (0.55-1.50)	1.12 (0.66-1.88)	45	0.75 (0.50-1.13)	0.92 (0.60-1.41)	91	1.00 (0.72-1.38)	1.28 (0.90-1.81)
P for trend			0.371	0.895		0.067	0.379		0.355	0.666
Orange/yellow vegetables and fruits (g/d)										
T1 (< 21.1)	416	28	1.00	1.00	47	1.00	1.00	86	1.00	1.00
T2 (21.1- < 54.0)	418	43	1.52 (0.93-2.50)	1.80 (1.08-3.01)	73	1.55 (1.05-2.29)	1.86 (1.24-2.80)	138	1.61 (1.19-2.18)	1.86 (1.34-2.59)
T3 (≥ 54.0)	416	42	1.49 (0.90-2.45)	1.94 (1.16-3.27)	58	1.23 (0.82-1.85)	1.60 (1.04-2.46)	96	1.12 (0.81-1.55)	1.47 (1.04-2.09)
P for trend			0.217	0.03		0.683	0.121		0.837	0.178

Orange / yellow fruits (g/d)										
T1 (< 15.9)	416	38	1.00	1.00	69	1.00	1.00	120	1.00	1.00
T2 (15.9-< 47.9)	417	46	1.20 (0.77-1.89)	1.43 (0.90-2.27)	61	0.88 (0.61-1.27)	1.04 (0.71-1.52)	115	0.96 (0.72-1.28)	1.11 (0.82-1.52)
T3 (≥ 47.9)	417	29	0.76 (0.46-1.25)	1.04 (0.62-1.75)	48	0.69 (0.47-1.02)	0.92 (0.61-1.38)	85	0.71 (0.52-0.96)	0.97 (0.69-1.36)
P for trend			0.155	0.849		0.065	0.625		0.02	0.724
Orange / yellow vegetable (g/d)										
T1 (< 2.7)	416	31	1.00	1.00	38	1.00	1.00	70	1.00	1.00
T2 (2.7-< 7.7)	417	27	0.87 (0.51-1.48)	1.01 (0.59-1.75)	55	1.44 (0.93-2.23)	1.68 (1.07-2.63)	101	1.44 (1.03-2.01)	1.64 (1.15-2.34)
T3 (≥ 7.7)	417	55	1.79 (1.12-2.86)	2.15 (1.32-3.51)	85	2.28 (1.51-3.44)	2.72 (1.77-4.18)	149	2.19 (1.59-3.02)	2.51 (1.77-3.54)
P for trend			0.003	< 0.001		< 0.001	< 0.001		< 0.001	< 0.001
White vegetables and fruits (g/d)										
T1 (< 87.9)	416	51	1.00	1.00	87	1.00	1.00	156	1.00	1.00
T2 (87.9- < 153.9)	418	46	0.88 (0.58-1.35)	1.04 (0.67-1.61)	68	0.77 (0.54-1.09)	0.89 (0.62-1.28)	100	0.63 (0.48-0.84)	0.76 (0.55-1.03)
T3 (≥ 153.9)	416	16	0.31 (0.17-0.55)	0.40 (0.22-0.73)	23	0.26 (0.16-0.42)	0.34 (0.21-0.56)	64	0.41 (0.29-0.56)	0.58 (0.41-0.83)
P for trend			< 0.001	0.003		< 0.001	< 0.001		< 0.001	0.002
Women										
Total vegetables and fruits (g/d)										
T1 (< 317.7)	199	29	1.00	1.00	64	1.00	1.00	70	1.00	1.00
T2 (317.7-< 534.8)	199	19	0.65 (0.35-1.20)	0.66 (0.35-1.25)	28	0.44 (0.27-0.71)	0.47 (0.28-0.79)	41	0.58 (0.38-0.90)	0.65 (0.41-1.03)
T3 (≥ 534.8)	198	5	0.17 (0.07-0.45)	0.22 (0.08-0.58)	21	0.33 (0.19-0.56)	0.41 (0.23-0.71)	13	0.19 (0.10-0.35)	0.25 (0.13-0.47)
P for trend			< 0.001	0.002		< 0.001	0.001		< 0.001	< 0.001
Total vegetables (g/d)										
T1 (< 135.9)	198	26	1.00	1.00	51	1.00	1.00	61	1.00	1.00
T2 (135.9-< 219.1)	200	22	0.83 (0.45-1.51)	1.02 (0.53-1.95)	41	0.80 (0.50-1.26)	0.97 (0.59-1.57)	45	0.73 (0.47-1.12)	0.82 (0.52-1.31)
T3 (≥ 219.1)	198	5	0.19 (0.07-0.50)	0.22 (0.08-0.61)	21	0.41 (0.29-0.71)	0.50 (0.28-0.89)	18	0.29 (0.17-0.51)	0.36 (0.20-0.65)
P for trend			< 0.001	0.003		0.001	0.018		< 0.001	< 0.001
Total fruits (g/d)										
T1 (< 135.0)	198	21	1.00	1.00	46	1.00	1.00	59	1.00	1.00
T2 (135.0-< 307.1)	199	27	1.29 (0.70-2.35)	1.37 (0.73-2.58)	46	1.00 (0.63-1.57)	1.05 (0.65-1.69)	48	0.81 (0.53-1.25)	0.88 (0.56-1.38)
T3 (≥ 307.1)	199	5	0.24 (0.09-0.64)	0.27 (0.10-0.76)	21	0.45 (0.26-0.79)	0.52 (0.29-0.93)	17	0.29 (0.16-0.51)	0.35 (0.19-0.64)
P for trend			0.003	0.002		0.004	0.006		< 0.001	< 0.001
Green vegetables and fruits (g/d)										
T1 (< 61.0)	199	26	1.00	1.00	60	1.00	1.00	60	1.00	1.00
T2 (61.0- < 114.2)	198	23	0.87 (0.48-1.59)	1.03 (0.55-1.93)	39	0.65 (0.42-1.02)	0.78 (0.49-1.26)	51	0.85 (0.56-1.29)	0.98 (0.62-1.54)
T3 (≥ 114.2)	199	4	0.15 (0.05-0.43)	0.18 (0.06-0.54)	14	0.23 (0.12-0.43)	0.28 (0.15-0.53)	13	0.21 (0.11-0.40)	0.26 (0.14-0.50)
P for trend			< 0.001	0.002		< 0.001	< 0.001		< 0.001	< 0.001
Red/purple vegetables and fruits (g/d)										
T1 (< 39.5)	199	20	1.00	1.00	41	1.00	1.00	47	1.00	1.00
T2 (39.5- < 98.5)	199	26	1.30 (0.70-2.41)	1.54 (0.81-2.95)	44	1.07 (0.67-1.71)	1.19 (0.72-1.95)	49	1.04 (0.67-1.63)	1.18 (0.73-1.89)
T3 (≥ 98.5)	198	7	0.35 (0.15-0.85)	0.43 (0.17-1.06)	28	0.69 (0.41-1.15)	0.75 (0.43-1.30)	28	0.60 (0.36-1.00)	0.71 (0.41-1.21)
P for trend			0.013	0.052		0.118	0.232		0.035	0.157
Orange/yellow vegetables and fruits (g/d)										
T1 (< 40.7)	199	16	1.00	1.00	33	1.00	1.00	40	1.00	1.00
T2 (40.7- < 100.6)	198	22	1.38 (0.70-2.71)	1.58 (0.78-3.20)	50	1.52 (0.94-2.47)	1.72 (1.03-2.87)	62	1.56 (1.00-2.43)	1.74 (1.08-2.80)
T3 (≥ 100.6)	199	15	0.93 (0.45-1.93)	1.05 (0.49-2.24)	30	0.91 (0.53-1.55)	0.98 (0.56-1.72)	22	0.55 (0.32-0.96)	0.61 (0.34-1.09)
P for trend			0.674	0.891		0.461	0.634		0.013	0.041

Orange/yellow fruits (g/d)										
T1 (< 32.5)	199	18	1.00	1.00	38	1.00	1.00	48	1.00	1.00
T2 (32.5- < 90.6)	199	22	1.22 (0.63-2.34)	1.44 (0.72-2.85)	47	1.24 (0.77-1.98)	1.43 (0.87-2.35)	56	1.17 (0.76-1.80)	1.35 (0.85-2.15)
T3 (≥ 90.6)	198	13	0.72 (0.35-1.52)	0.79 (0.37-1.70)	28	0.74 (0.44-1.25)	0.77 (0.44-1.35)	20	0.42 (0.24-0.73)	0.44 (0.25-0.80)
P for trend			0.281	0.37		0.15	0.198		< 0.001	0.002
Orange/yellow vegetable (g/d)										
T1 (< 3.2)	198	13	1.00	1.00	27	1.00	1.00	34	1.00	1.00
T2 (3.2- < 9.1)	200	11	0.84 (0.37-1.91)	1.17 (0.49-2.75)	28	1.03 (0.59-1.81)	1.35 (0.75-2.46)	28	0.82 (0.48-1.40)	1.04 (0.59-1.84)
T3 (≥ 9.1)	198	29	2.21 (1.11-4.40)	2.87 (1.36-6.03)	58	2.20 (1.33-3.64)	2.54 (1.48-4.36)	62	1.85 (1.16-2.95)	2.05 (1.24-3.40)
P for trend			0.005	0.002		< 0.001	< 0.001		0.001	0.002
White vegetables and fruits (g/d)										
T1 (< 109.9)	199	31	1.00	1.00	60	1.00	1.00	61	1.00	1.00
T2 (109.9- < 198.0)	199	15	0.49 (0.25-0.93)	0.59 (0.30-1.16)	36	0.60 (0.38-0.95)	0.75 (0.46-1.22)	45	0.74 (0.48-1.14)	0.93 (0.59-1.48)
T3 (≥ 198.0)	198	7	0.22 (0.10-0.50)	0.25 (0.10-0.59)	17	0.28 (0.16-0.50)	0.35 (0.19-0.63)	18	0.30 (0.17-0.52)	0.37 (0.21-0.67)
P for trend			0.001	0.002		< 0.001	0.001		< 0.001	< 0.001

[1]Adjusted by age, education, alcohol consumption, BMI, regular exercise, red meat, processed meat and total energy intake; [2]Test for trend calculated with the median intake for each category of vegetables and fruits as a continuous variable.

consumption can contribute to the development of melanoma[44]. Citrus is rich in psoralens and furocoumarins, which raise melanoma risk through photocarcinogens. However, negative health effects of psoralens and furocoumarins on colorectal cancer have not been found in epidemiologic studies. Therefore, we cannot explain the association and possible mechanism for the increased risk of colorectal cancer.

This study shows that high orange/yellow vegetables intake elevates the risk of colorectal cancer. Orange/yellow vegetables include carrot, pumpkin, and ginger. In a case control study from Western Australia[12] and Prostate, Lung, Colorectal and Ovarian (PLCO) cancer screening trial study[45] reported the protective effects of dark yellow vegetables (carrot, pumpkin) for colorectal cancer risk. Gingerol and supplementation with ginger root extract inhibit colorectal carcinoma progress in vivo and humans[46,47]. However, safrole, ingredients that generated when ginger rotted, and group 2B carcinogen classified by the IARC[48], is known to induce cancer in rodents[49,50]. Also, the remaining chemical additives (fertilizer, preservatives, pesticide) after washing are likely to cause cancer. We have no definite explanation that orange/yellow vegetables intake increase the risk of developing colorectal cancer. More research is needed to verify this observation.

Our results showed a sex difference. Although the underlying mechanism for the sex difference of our study between sexes is not clearly known, few possibilities can be considered from various aspects. Previous studies have suggested that estrogen exposure[51] and the use of oral contraception[52] prevented the development of colorectal cancer. Also, taking hormonal replacement therapy (HRT) in postmenopausal women showed reduced colorectal cancer risks in Women's Health Initiative (WHI) study[53] and a meta-analysis[54]. Another reason is that women tend to prefer vegetables and fruits than men. Because usually women are responsible in buying and cooking foods in Korean culture, they tend to have more information about beneficial health effects of vegetable/fruits and consume more of them[55]. Other factors such as prevalence of diabetes, physical activity, education and income levels, and lifestyle differences between sexes may influence the relationship between vegetables and fruits intake and colorectal cancer risk.

Korean diet has a unique synchronic serving method/style of which all dishes are served at one time on a table. On the other hand, Western or Chinese diet is diachronic (course meal), serving dishes at different points of time[56]. A Typical and common Korean table is set with bap (steamed rice), kuk or chigae (broth, stew), banchan (side dishes) and kimchi[57]. Bap is the main Korean dish that gives a major source of energy. Kuk or chigae, which are different than the Western soups[58] are eaten with bap. Usually, banchan (side dishes) are composed of more than three kinds of foods such as namul, legumes, fish, meat, and kimchi, and are seasoned with jang, sesame or perilla seed/oil, vinegar, and herbs. Korean diet is usually well-balanced and nutritious. Based on these features, the health benefits of the Korean diet are reported in many cases of diseases[59,60]. Currently, peoples believe that colorful vegetables and fruits are the most nutritious and indicate that the distinction between the color of vegetables and fruits is a powerful factor in food selection[61]. The information presented in this study could be used to advise members of the general Korean public who are interested in the phytochemicals of vegetables and fruits. However, it is difficult to generalize to the population of many countries in the

world. Because each country has its own traditional recipe and the unique vegetables and fruits that are naturally grown in each climate and topography.

The present study has several limitations. First, because the design of our study relied on hospital-based case-control groups, and the control group was recruited from participants in the health check-up program of the National Health Insurance Corporation, the results of our study may not be representative of the source population of the cases[62]. The control group could have had healthy behaviors and habits compared to the patients. Second, recall bias is an inherent weakness in case-control study design. Case and control groups tend to have differences in recall. Colorectal cancer patients are likely to overestimate or underestimate their poor eating habits compared to the control group[25]; therefore, there is the possibility of exaggerating of the association. To reduce this problem, we tried to survey the case group as soon as their cancer was diagnosed or just before surgery. Third, we did not evaluate the manufacturing method (cooked, raw, or frozen) or extra ingredients (seasoning, dressing, *etc*.). The majority of study suggested that the inverse relationship for cancer may be stronger for raw vegetables, in which destruction of nutrients is minimized compared to cooked vegetables. But, compared to other cancers, colorectal cancer showed similar results between raw vegetables and cooked vegetables[63]. Lastly, we could not further consider the molecular characteristics such as microsatellite Instability or CpG island methylator phenotype of colorectal cancer patients, which could be related with differential risk.

In conclusion, our results suggest that total vegetables and fruits intake by color was inversely related to colorectal cancer risk. However, the orange/yellow vegetables and fruits color group showed an elevated risk for colorectal cancer. Further studies are necessary to confirm the relationship between vegetable and fruit intake by color and colorectal cancer risk.

COMMENTS

Background

The colors of vegetable and fruit reflect their contents of unique phytochemicals and micronutrients. In this case-control study, the authors investigated the relationship between the colors of vegetable and fruit and the risk of colorectal cancer.

Research frontiers

The authors conducted a case-control study to investigate the association between the vegetable and fruit color group and colorectal cancer risk in the Korea population.

Innovations and breakthroughs

Methods that classify vegetables and fruits by color are rare in most studies of disease including colorectal cancer. Vegetables and fruits that are consumed by Koreans were classified according to the criteria.

Applications

Results of this study may be used to advise the general Koreans who are interested in prevention of colorectal cancer.

Terminology

The color of vegetables and fruits reflect the contents of unique phytochemicals and micronutrients. Vegetables and fruits intake in various color groups may protect against colorectal cancer.

Peer-review

Presented manuscript depicts interesting way of seeing of diet-factors impact to colorectal cancer genesis. Discrimination of vegetables and fruits according to only their colour and hypothetical natural consent is substantially difficult in light of reliable statistical analysis. However, there are consistent preventive data of cruciferous vegetables, garlic or fiber-rich plants. The meaning of achieved results should be very careful. Available vegetables and fruits include diversified values of chemical additives, various preservatives and chemical fertilizers as well. Vast used, *e.g.,* to citrus preservation, fungicides such as enilkonasol and also tiabendasol have documented pro-cancerous action. Because of that, estimation of influence of dietary plants to cancer is especially difficult in the age of chemically modified plants.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Impact of vitamin D on the hospitalization rate of Crohn's disease patients seen at a tertiary care center

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Author contributions: Venkata KVR, Arora SS and Malik TA conceptualized the study hypothesis, design and methodology; Venkata KVR and Arora SS collected data by retrospective chart review; Xie FL performed the statistical analysis; With regard to manuscript write up, Venkata KVR compiled the methods and results section, while Arora SS and Malik TA wrote the introduction and discussion section and all authors proof read for final manuscript edits.

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Abstract

AIM

To study the association between vitamin D level and hospitalization rate in Crohn's disease (CD) patients.

METHODS

We designed a retrospective cohort study using adult patients (> 19 years) with CD followed for at least one year at our inflammatory bowel disease center. Vitamin D levels were divided into: low mean vitamin D level (< 30 ng/mL) *vs* appropriate mean vitamin D level (30-100 ng/mL). Generalized Poisson Regression Models (GPR) for Rate Data were used to estimate partially adjusted and fully adjusted incidence rate ratios (IRR) of hospitalization among CD patients. We also examined IRRs for vitamin D level as a continuous variable.

RESULTS

Of the 880 CD patients, 196 patients with vitamin D level during the observation period were included. Partially adjusted model demonstrated that CD patients with a low mean vitamin D level were almost twice more likely to be admitted (IRR = 1.76, 95%CI: 1.38-2.24) compared to those with an appropriate vitamin D level. The fully adjusted model confirmed this association (IRR = 1.44, 95%CI: 1.11-1.87). Partially adjusted model with vitamin D level as a continuous variable demonstrated,

higher mean vitamin D level was associated with a 3% lower likelihood of admission with every unit (ng/mL) rise in mean vitamin D level (IRR = 0.97, 95%CI: 0.96-0.98). The fully adjusted model confirmed this association (IRR = 0.98, 95%CI: 0.97-0.99).

CONCLUSION

Normal or adequate vitamin D stores may be protective in the clinical course of CD. However, this role needs to be further characterized and understood.

Key words: Crohn's disease; Vitamin D; Vitamin D deficiency; Hospitalization rate; Inflammatory bowel disease

Core tip: Growing body of epidemiological evidence supports a key role of vitamin D deficiency not just in inflammatory bowel disease development but also on Crohn's disease (CD) severity. Our study sought to test the hypothesis that adequate vitamin D levels have a protective role in the clinical course of CD in terms of a decreased likelihood of hospitalization. Our results are clinically important as they suggest potentially worse outcomes in CD patients with low vitamin D levels as reflected by a numerically increased rate of hospitalization in this group.

Venkata KVR, Arora SS, Xie FL, Malik TA. Impact of vitamin D on the hospitalization rate of Crohn's disease patients seen at a tertiary care center. *World J Gastroenterol* 2017; 23(14): 2539-2544 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i14/2539.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i14.2539

INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory disorder characterized by transmural inflammation (all layers from mucosa to serosa) that may discontinuously involve any part of the alimentary tract[1-4]. First described by Crohn *et al*[1] in 1932, 750000 people in the United States currently have CD. It is classified as inflammatory, penetrating, or stricturing, with or without perianal disease[5,6]. As CD became recognized as a distinct disease entity, it was observed that vitamin D deficiency was common among these patients[7,8].

Vitamin D exerts immune modulatory effects by reducing T cell mediated up-regulation of the nuclear vitamin D receptor (VDR)[9,10]. The gene for VDR signals through enhancer segments in the *NOD2* gene, thereby inducing NF-kappaB transcription factor function. This in turn stimulates gene encoding antimicrobial peptide defensin beta2 (DEFB2/HBD2). However, this sequential activation is absent in macrophages of CD patients thus favoring intestinal inflammation[11]. Further, certain VDR gene polymorphisms such as rs731236[A] (VDR) and rs732594[A] (SCUBE3) have been found to directly influence risk of CD[12]. A 2013 meta-analysis showed that carrying "Taql tt" genotype of the VDR gene is associated with increased susceptibility for CD in Europeans, while Apal "a" allele is protective. Therefore[13], vitamin D is believed to play an integral role in immune pathogenesis of CD and may help reduce CD-related hospitalizations, disease severity, need for surgery, and colon cancer incidence[14,15].

Growing body of epidemiological evidence supports a key role of vitamin D deficiency not just in inflammatory bowel disease (IBD) development but also on CD severity[16,17]. Studies suggest association between low vitamin D levels and increased disease activity as reflected by fecal calprotectin levels[13,18], hospitalizations as well as need for surgery in CD patients[15,19]. Conversely, vitamin D supplementation in CD may reduce chronic intestinal inflammation as reflected by CD activity index (CDAI) and C-reactive protein levels[20-22], as well as relapse frequency by as much as 50%[18].

University of Alabama at Birmingham (UAB) is the only tertiary care IBD referral center in the state of Alabama that provides health care by dedicated sub-specialists in a large hospital with sophisticated intensive care facilities after referral from primary care and smaller hospitals. IBD center has facilities available for both inpatient and outpatient management of patients with CD and its complications and so it is a unique setting to study the effect of various covariates such as vitamin D levels on outcomes in CD. Our study sought to test the hypothesis that adequate vitamin D levels have a protective role in the clinical course of CD in terms of a decreased likelihood of hospitalization.

MATERIALS AND METHODS

Study design, patient population, and selection criteria

We conducted a retrospective cohort study to look at vitamin D levels and CD outcomes. For this study, we analyzed data from 880 CD patients seen at our tertiary care IBD center from 2000 to 2014 and followed for at least one year. Subjects were included in the analysis if they were older than 19 years and had vitamin D levels available. Other included variables were duration of disease, race, sex, smoking status, use of steroids, biological agents, thiopurines or methotrexate and hospitalization rate. The University of Alabama's Office of Institutional Review Board (IRB) approved the study and it was deemed compliant with the Helsinki declaration.

Data collection and variable definitions

Data were collected by means of retrospective chart review, specifically per Electronic medical record (EMR) documentation and laboratory results. Data collected at the time of first observation

Table 1 Characteristics of Crohn's disease patients by mean vitamin D level

	Mean < 30 (*n* = 115)	Mean ⩾ 30 (*n* = 81)
Age, (mean ± SD, yr)	45.50 (15.07)	54.26 (17.63)
DoD	17.83 (11.77)	22.58 (14.50)
Race		
Caucasian	66.96%	87.65%
African-American	31.30%	12.35%
Others	1.74%	0.00%
Female	61.74%	71.6%
BMI		
Low (< 18.5)	11.30%	8.64%
Normal (18.5-24.9)	40.00%	41.98%
Over Weight (25-29.9)	20.00%	27.16%
Obese (⩾ 30)	28.70%	22.22%
Smoking	26.96	11.11
Steroids	51.30	45.68
Immune modulators	84.35%	76.54%
Biologicals	61.74%	51.85%
Thiopurines	61.74%	51.85%
Methotrexate	20.87	13.58

DoD: Duration of disease; BMI: Body mass index.

included age, race, sex, duration of CD and vitamin D levels. Participants were followed through the last observation at our IBD center for CD-related hospitalizations. We also collected data on body mass index (BMI), smoking history, medication history for steroid use, traditional and biological immune modulator use. Steroid use was defined as exposure to oral or parenteral corticosteroids for at least six weeks during observation. Thiopurine use was defined as use of azathiopurine or 6-mercaptopurine for at least four weeks during the period of observation. Methotrexate (MTX) use was defined as use of MTX for at least four weeks during the period of observation. Biologic use was defined as use of any biologic agent for at least four weeks during the period of observation. A CD-related hospitalization was defined as any hospital admission for a complication of CD, including infections, fistula, strictures, abscess or exacerbations. 25-Hydroxy vitamin D concentration was measured by Immunoassay method. Adequate vitamin D level was 30-100 ng/mL, while vitamin D level < 30 ng/mL was considered low. We used 30ng/ml as threshold as it is the laboratory reference value for normal lower limit of vitamin D levels in our hospital.

Statistical analysis

After calculating summary statistics, we performed univariate analyses to examine the incidence rates of CD related hospitalizations among CD patients based on vitamin D levels. We then built Generalized Poisson Regression Models for rate data to estimate partially adjusted (for age, sex, race and duration of disease) as well as fully adjusted (additionally for BMI, smoking, steroid use, traditional and biological immune modulator use) incidence rate ratios (IRR) of hospitalization among CD patients with low mean vitamin D levels (< 30 ng/mL) *vs* those with adequate mean vitamin D levels (30-100 ng/mL) during the entire follow up (observation) period.

For each patient, the period of observation was defined as the time in years between the first and the last documented encounter at our tertiary care IBD center during the years 2000 through 2014. All statistical analyses were conducted using SAS, version 9.4 (SAS Institute Inc., Cary, NC). Statistical tests were two-sided with a significance level alpha < 0.05.

RESULTS

Vitamin D levels were measured in 196 of 880 CD patients seen at our institute during the observation period and were included in this study. Of these, 115 patients had a low mean vitamin D level and 81 had an appropriate vitamin D level (Table 1). Among CD patients, incidence rate of hospitalization for a CD related exacerbation was 30.18 per 100 person-years with low mean vitamin D level *vs* 14.19 per 100 person-years with an appropriate mean vitamin D level (Table 2). GPR Model for Rate Data that was partially adjusted demonstrated that CD patients with a low mean vitamin D level were 1.76 times more likely to be admitted during the observation period (IRR = 1.76, 95%CI: 1.38-2.24) compared to those with an appropriate vitamin D level. The fully adjusted (adjusted for age, sex, duration of CD, smoking, BMI and CD therapy) model confirmed this clinically and statistically significant association (IRR = 1.44, 95%CI: 1.11-1.87) (Table 2).

Partially adjusted (adjusted for age, sex, race, duration of disease) GPR Model for Rate Data with vitamin D level as a continuous variable, demonstrated that higher mean vitamin D level was associated with a lower likelihood of admission with every unit (ng/mL) rise in mean vitamin D level associated with a 3% lower risk of admission during the observation period (IRR = 0.97, 95%CI: 0.96-0.98). The fully adjusted model confirmed this clinically and statistically significant association (IRR = 0.98, 95%CI: 0.97-0.99).

DISCUSSION

We demonstrated that CD patients with a low mean vitamin D level (< 30 ng/mL) were almost 1.5 times more likely to be admitted (IRR = 1.44, 95%CI: 1.11-1.87) compared to those with an appropriate vitamin D level. Overall, the likelihood of CD-related hospitalization decreased by about 3% with every unit (ng/mL) rise in mean vitamin D level. Our findings could have a few plausible interpretations: (1) Vitamin D may serve as a surrogate marker of CD severity in terms of general ill-state, CD activity or exacerbations meriting hospitalization and the need for surgery; (2) CD patients may be more likely to be admitted if they have low vitamin D levels compared to those with

Table 2 Crude, partially adjusted, fully adjusted rate ratios for Crohn's disease- related hospitalization

	CD-related number of hospitalizations/total person years	Hospitalization rate (95%CI)[1]	IRR (95%CI)		
			Model 1	Model 2	Model 3
Overall	372/1610	23.11 (20.87, 25.58)			
Mean Vitamin D level ⩾ 30 mg/dL	101/712	14.19 (11.67, 17.24)	1 (reference)	1 (reference)	1 (reference)
Mean Vitamin D level < 30 mg/dL	271/898	30.18 (26.79, 33.99)	2.13(1.69, 2.67)	1.76 (1.38, 2.24)	1.44 (1.11, 1.87)

[1]Per 100 person-year. Model 1 is unadjusted; Model 2 is partially adjusted for age, sex, race, duration of disease; Model 3 is fully adjusted for age, sex, race, duration of disease, BMI, smoking, steroids, traditional and biological, immune modulators, thiopurines, methotrexate. CD: Crohn's disease; BMI: Body mass index.

adequate vitamin D levels, despite same degree of CD activity.

Of note, our study results are in agreement with prior studies that normal or adequate vitamin D stores may play a protective role in the clinical course of CD[14,21]. Furthermore, when adjusted for covariates including age, sex, race, duration of disease, BMI, smoking, steroid use, traditional and biological immune modulator use; the disparity in CD-related hospitalization rate remained significant among the two vitamin D groups. This striking difference in observed admission rates indeed warrants further investigation to further characterize and understand the role of vitamin D in CD.

Several factors have been shown to predict vitamin D deficiency in CD. These include: insufficient sunlight exposure, malnutrition, impaired conversion of vitamin D to metabolite (*i.e.*, 25-hydroxycholecalciferol), accelerated breakdown, heightened excretion, and gene mutations affecting vitamin D hydroxylation and transport[22-24]. Besides, a notable seasonal variation has been observed in CD in form of a winter decline in vitamin D levels and rise in bone turnover markers such as serum parathyroid hormone, osteocalcin, bone-specific alkaline phosphatase and urinary N-telopeptides of type 1 collagen[25]. Meanwhile, non-Caucasian ethnicity, adequate sun exposure and avoidance of tanning beds have been found to be associated with sufficient vitamin D levels in CD[26].

CD might itself be the root of vitamin D deficiency. Inflammatory cytokines in CD suppress renal 1-alpha hydroxylase leading to vitamin D deficiency[27,28]. Furthermore, CD is associated with altered T cell response to gut microflora. Emerging evidence from animal studies has linked vitamin D deficiency to T cell self-reactivity and loss of immune tolerance to self-structures[29]. Longer disease duration, CD disease activity and smoking status inversely correlate with serum vitamin D levels[22,30].

The Endocrine Clinical Practice Guidelines Committee recommends screening of all IBD patients especially those on corticosteroids for vitamin D status[31]. Among CD patients, serum vitamin D levels must be assessed especially for those with: elevated ESR[8], long duration of CD (> 15 years) and extended active stage of disease[32]. Between the two vitamin D subtypes, the active form of vitamin D (*i.e.*, 25-hydroxycholecalciferol) has more marked beneficial effect on CD activity as reflected by decrease in C-reactive protein levels[33]. Further, oral active vitamin D is better absorbed even in presence of distal small-bowel resection in CD, and should therefore be preferred to cholecalciferol, especially in CD patients with severe short-bowel syndrome[34].

Among potential limitations of our study, the following are noteworthy. We accounted for CD-related hospitalizations exclusively within our institution. Furthermore, we studied a small proportion of CD patients seen at our institution, *i.e.*, those with vitamin D levels drawn. This could have potentially led to selection bias. Retrospective observational study design and the use of EMR for data extraction are additional limitations. Due to this limitation, we couldn't accurately assess the various causes associated with vitamin D deficiency in our patient population. Although vitamin D levels fluctuate in various seasons possibly due to difference in day light sun exposure, we did not differentiate vitamin D levels according to the season, as we assumed that state of Alabama has adequate day light sun exposure throughout the year relative to the North-eastern and Mid-western United states. We calculated mean values for vitamin D levels collected throughout our observation period. This would balance variation in vitamin D levels around the year when represented as a normal distribution.

In regard to whether our study's conclusions are generalizable to all CD patients, one should bear in mind that the segment of CD patients seen at our tertiary care IBD referral center represents those with a more severe disease phenotype. This may explain the significantly higher overall CD hospitalization rate within our study population. Our findings are in general applicable and relevant to CD patients with moderate to severe disease compared to those with mild CD

While previous papers have studied the association between vitamin D and clinical disease activity in CD, our study is unique as it examines the association between Vitamin D levels and Crohns related hospitalization rates[17,18]. This association merits further investigation because vitamin D is a modifiable risk factor. Vitamin D level may serve as a potential therapeutic and a health maintenance target to improve quality of life and reduce complications in CD. Further studies need to be done to assess if

interventions to raise Vitamin D level will decrease hospitalization rates. Also future research on this topic should consider looking at the association between vitamin D levels and other markers of disease outcome in Crohn's such as need for surgery and the frequency and duration of corticosteroid use as well as mean disease activity parameters through observation.

COMMENTS

Background

As Crohn's disease (CD) became recognized as a distinct disease entity, it was observed that vitamin D deficiency was common among these patients. Vitamin D is believed to play an integral role in immune pathogenesis of CD and may help reduce CD-related hospitalizations, disease severity, need for surgery, and colon cancer incidence. Growing body of epidemiological evidence supports a key role of vitamin D deficiency not just in inflammatory bowel disease development but also on CD severity. This study sought to test the hypothesis that adequate vitamin D levels have a protective role in the clinical course of CD in terms of a decreased likelihood of hospitalization.

Research frontiers

Recent meta-analysis and other studies showed association between vitamin D and CD. The authors provide support to hypothesis with this paper, reporting decreased likelihood of hospitalization in CD patients with adequate vitamin D level.

Innovations and breakthroughs

This paper shows that low vitamin D levels are associated with potentially worse outcomes in CD patients as reflected by a numerically increased rate of hospitalization in this group.

Applications

Patients with low vitamin D levels are associated with increased hospitalization rate but further studies needs to be done to assess if intervention to raise vitamin D levels will decrease hospitalization rates.

Terminology

A CD-related hospitalization was defined as any hospital admission for a complication of CD, including infections, fistula, strictures, abscess or exacerbations.

Peer-review

This is a well-written manuscript on impact of adequate levels of Vit-D on hospitalization rates in patients with CD. The study is observational, based on retrospective chart review.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Barcelona clinic liver cancer nomogram and others staging/scoring systems in a French hepatocellular carcinoma cohort

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Abstract

AIM

To compare the performances of the Barcelona clinic liver cancer (BCLC) nomogram and others systems (BCLC, HKLC, CLIP, NIACE) for survival prediction in a large hepatocellular carcinoma (HCC) French cohort.

METHODS

Data were collected retrospectively from 01/2007 to 12/2013 in five French centers. Newly diagnosed HCC patients were analyzed. The discriminatory ability, homogeneity ability, prognostic stratification ability Akaike information criterion (AIC) and C-index were compared among scoring systems.

RESULTS

The cohort included 1102 patients, mostly men, median age 68 [60-74] years with cirrhosis (81%), child-Pugh A (73%), alcohol-related (41%), HCV-related (27%). HCC were multinodular (59%) and vascular invasion was present in 41% of cases. At time of HCC diagnosis BCLC stages were A (17%), B (16%), C (60%) and D (7%). First line HCC treatment was curative in 23.5%, palliative in 59.5%, BSC in 17% of our population. Median OS was 10.8 mo [4.9-28.0]. Each system distinguished different survival prognosis groups (P < 0.0001). The nomogram had the highest discriminatory ability, the highest C-index value. NIACE score had the lowest AIC value. The nomogram distinguished sixteen different prognosis groups. By classifying unifocal large HCC into tumor burden 1, the nomogram was less powerful.

CONCLUSION

In this French cohort, the BCLC nomogram and the NIACE score provided the best prognostic information, but the NIACE could even help treatment strategies.

Key words: Barcelona clinical liver cancer; Hong kong liver cancer; NIACE; CLIP; Hepatocellular carcinoma

Core tip: Barcelona clinic liver cancer (BCLC) nomogram was compared with BCLC, HKLC systems, CLIP, and NIACE scores for survival prediction in a HCC French cohort. 1102 patients were retrospectively included, with cirrhosis (81%), child-Pugh A (73%). Hepatocellular carcinoma (HCC) were multinodular (59%) and with vascular invasion (41%). At time of HCC diagnosis, patients were mainly BCLC-C (60%). First line HCC treatment was curative (23.5%) or palliative (59.5%). Median OS was 10.8 mo [4.9-28.0]. BCLC nomogram had the highest discriminatory ability, the highest C-index value. NIACE score had the lowest akaike information criterion value. In this French cohort, BCLC nomogram and NIACE score provided the best prognostic information.

Adhoute X, Pénaranda G, Raoul JL, Edeline J, Blanc JF, Pol B, Campanile M, Perrier H, Bayle O, Monnet O, Beaurain P, Muller C, Castellani P, Le Treut YP, Bronowicki JP, Bourlière M. Barcelona clinic liver cancer nomogram and others staging/scoring systems in a French hepatocellular carcinoma cohort. *World J Gastroenterol* 2017; 23(14): 2545-2555 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i14/2545.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i14.2545

INTRODUCTION

Survival prediction and therapeutic strategy for hepatocellular carcinoma (HCC) are based on Barcelona classification of liver cancer staging system (BCLC) in the West[1,2]. It has become the reference classification by its prognostic value, its simplicity, and its treatment algorithm based on randomized clinical studies[3]. However, HCC staging systems remain a controversial issue. Asian countries, in which HCC is mainly related to HBV, have their own staging systems and therapeutic recommendations[4]. The BCLC system has been criticized; the major issue is that stages B and C HCC include a broad spectrum of tumors with a single therapeutic option[5-7], and for some authors other treatments are possible[8-11]. Subsequently, changes have been made compared to the initial version of the BCLC system[12] with the transfer of single and large HCC > 50 mm from intermediate to early stages[3], enhancing the heterogeneity within this group[13]. Older scores such as CLIP[14] showed a better prognostic value than the BCLC system in large Asian and Western HCC cohorts[15,16]. Therefore, a new classification has been proposed, the HKLC system[17], which offers another stratification, and new therapeutic proposals with surgery and chemoembolization to treat more advanced HCC. Other scores, independent of the BCLC system[7,18,19] or additional to the BCLC system[20,21] have been proposed in recent years. NIACE score (tumor Nodularity, Infiltrative nature of the tumor, serum Alpha-fetoprotein level, Child-Pugh stage, ECOG performance status)[22] determines subgroups of different survival prognosis irrespective of the BCLC stage[23], or HCC treatment modalities[24]. This score has been validated either in European or Asian cohorts[25,26]. Recently, Hsu *et al*[27] proposed a simple nomogram, determined from a large HCC cohort mainly related to HBV in order to improve the prognostic value of the BCLC system.

The aims of this study were to assess and compare the performances of the BCLC nomogram and others staging and scoring systems (BCLC, HKLC, CLIP and NIACE) for survival prediction in a large European multicenter HCC cohort.

MATERIALS AND METHODS

This retrospective study was conducted in five French centers (Marseille, Nancy, Bordeaux, and Rennes). During a period of seven years, from January 2007 to December 2013, all HCC patients treated or not, have been included in this study.

HCC diagnosis was based on the identification of the typical hallmark of HCC (EASL - AASLD criteria)[28] and, if a patient did not have a typical HCC on imaging or a cirrhotic liver, or if there was discordant results between non-invasive criteria (such as fibrometer and fibroscan), a biopsy was required. The analyzed data (clinical, biological, radiological, therapeutic options, response to treatment and follow-up) were prospectively collected and retrospectively analyzed using the same methodology in the different centers. This study was approved by local ethics committee.

HCC were ranked at diagnosis and during follow-up according to their morphologies (nodular or infiltrative HCC) assessed by multi-sliced contrast-enhanced CT and/or MRI. Liver cancers were either nodular HCC, that is an arterially enhancing mass with clear demarcation and washout in the portal venous phase, or infiltrative HCC, that is an ill-defined tumor with no distinct margination of any portion, characterized by inhomogeneous areas of enhancement on the arterial phase images and corresponding areas of washout on more delayed phases of contrast enhancement. These tumors may be more visible among the surrounding liver parenchyma at diffusion- and T2- weighted MR images and are frequently associated with vascular invasion[29-32]. Early (BCLC A) and intermediate (BCLC B) HCC without vascular invasion, considered as infiltrative tumor as opposed to encapsulated tumors, were tumor with non-smooth tumor margins (*i.e.,* tumor with focal extranodular extension beyond the tumor capsule or focal infiltrative margin), or those with peritumoral enhancement[33-35], or those associated with biliary dilatation. Two liver imaging "senior experts" radiologists reviewed images retrospectively.

Patients' classification according to staging and scoring systems

Following categories were used for the BCLC classification: BCLC A HCC was defined as patients having solitary tumor > 2 cm or no more than 3 tumors not exceeding 3 cm in diameter, PS 0, Child-Pugh grade A or B.

BCLC B HCC encompassed patients with multiple tumors beyond 3 cm, PS 0, Child-Pugh grade A or B.

BCLC C encompassed any tumor with radiologically evident or histologically proven macrovascular invasion (portal vein, hepatic vein, inferior vena cava) and/or patients with lymph nodes and/or distant metastases and/or patients with cancer related - symptoms, with preserved liver function.

BCLC D encompassed tumors leading to a very poor performance status (PS 3-4), or patients with severe liver impairment (Child-Pugh B9 grade) and tumors beyond the transplantation threshold. Child-Pugh C patients were excluded because the NIACE score did not incorporate Child-Pugh C grade.

The HKLC classification, the CLIP score and the BCLC nomogram were applied to each patient before treatments initiation.

The NIACE score was calculated with all parameters collected before treatments initiation, as follows: 1x (Nodular numbers 0 if < 3, 1 if $\geqslant$ 3) + 1.5x (Infiltrating tumors: 0 if no, 1 if yes) + 1.5x (Alpha-fetoprotein level: 0 if < 200, 1 if $\geqslant$ 200 ng/mL) + 1.5x (Child-Pugh grade: 0 if A, 1 if B) + 1.5x (ECOG PS score 0 if 0, 1 if $\geqslant$ 1).

Treatments

Treatment and follow-up modalities were applied similarly in all centers.

Surgery: In general, patients with resectable tumors were selected for surgery if they had a performance status of 0 with both Child-Pugh grade A or B7, and on the basis of their functional hepatic reserve (indocyanine green retention rate at 15 min < 15%) and on the estimated remnant liver volume, regardless of HCC morphologies. Our protocols for the assessment of FHR and determination of surgical extent include biochemical liver function tests, blood cell count, IGR R15, and triphasic liver CT with volumetry. Gastroesophageal endoscopic findings were also taken into consideration for cirrhotic livers.

Patients without clinically significant portal hypertension and with normal serum bilirubin value were first considered for resection. Patients who underwent surgery *vs* radiofrequency ablation were as expected younger with less cirrhosis and larger tumor size. In cirrhosis, candidates for resection were carefully selected to diminish the risk of post-operative liver failure[36]. Portal hypertension (presence of either esophageal varices (EV), or splenomegaly with platelet count below 100000/mm^3) was considered as a contraindication for liver resection, but in BCLC A HCC patients with well-preserved liver function, and IGR at 15 min < 15%, not suitable for radiofrequency ablation (RFA) or transplantation, a minor hepatic resection was proposed[37-39]. Surgery was made after endoscopic treatment of EV.

Some BCLC C HCC patients were selected for hepatectomy according to the following selection criteria: PS 0, Child-Pugh A with bilirubin level $\leqslant$ 1.0 mg/dL, single nodule with limited portal vein thrombosis (*i.e.,* with second-order branch and third-order branch)[8].

Radiofrequency ablation: Applied in patients with resectable tumor $\leqslant$ 50 mm of diameter or within the Milan criteria (single tumor $\leqslant$ 50 mm or up to three

tumors ⩽ 30 mm in diameter).

Patients who underwent both radiofrequency ablation and chemoembolization *vs* radiofrequency ablation alone had larger tumor size.

Chemoembolization: Multinodular HCC with enhancing lesions, PS 0, Child-Pugh grade A or B7, were treated by TACE, regardless of HCC morphologies. Patients were treated by conventional TACE using the same inclusion/exclusion criteria in the different centers. TACE (Trans Arterial Chemoembolization) was performed in a standard fashion with a selective injection of a mixture of epirubicin (50 mg) and lipiodol (10 mL), followed by embolization with Gelfoam fragments. A second TACE was carried out 6 to 8 weeks later unless clear progress or serious adverse events occurred. Other TACE procedures were planned "on demand", according to the results of radiological and AFP assessments made every 12 wk. The EASL criteria, based on a bi-dimensional measurement of the tumor's enhanced viable component, were used to evaluate tumor response[40,41].

Patients with segmental vein thrombosis were left in the analysis because, in most centers, this is not considered as a contraindication for TACE[42,43].

Patients excluded from this retrospective analysis were: patients who received TACE as a bridge for liver transplantation; Child-Pugh C patients, and patients treated by liver transplantation.

Sorafenib

The initial sorafenib dose was determined according to different factors, such as Eastern Cooperative Oncology Group Performance Status and liver function. Child-Pugh A patients received 400 mg twice a day and Child-Pugh B patients 200 mg, twice a day. A reduction in the sorafenib dose or a temporary interruption was allowed, depending on the type and severity of any adverse event (grade 2 or higher on the National Cancer Institute's Common Terminology Criteria for Adverse Events (CTCAE), version 4.0). Sorafenib treatment was continued unless intolerable toxicity or clinical disease progression was observed. CT and/or MRI were used to evaluate the tumor response every 3 mo.

Patients had received Sorafenib since 2008; fifty six patients had received other palliative treatments before 2008 including tamoxifen or pravastatin (n = 23), or chemotherapy with doxorubicin (n = 20), and others drugs in clinical trials (n = 13).

Statistical analysis

Continuous data are expressed as median [quartile 1 - quartile 3] and categorical data are expressed as rates. Normality of the data was assessed by Shapiro-Wilks test. Overall survival was the endpoint used. The time of survival was defined as the time interval between the diagnosis of hepatocellular carcinoma and death or time of last follow-up. Proportionality of the subdistribution hazards was assessed by both inspecting Schoenfeld-type residuals and testing correlation of these residuals with time[44]. In case of proportionality of hazards across time, survivals between groups were compared using log-rank test; generalized Wilcoxon test was used in case on non-proportionality of hazards[45]. Discriminatory ability of each staging system was performed using χ^2 linear trend test (LT) and the Akaike information criteria (AIC): the higher is the LT and the lower is the AIC, the higher is the discriminatory ability of the model. Homogeneity of each staging system was performed using likelihood ratio (LR) calculated using the Cox regression model: the higher de LR, the lower is the difference among the patients classified into the same group by each staging system. The C-index was also used to determine the performance of the model. The larger the C-index, the more accurate the prognostic prediction was[46]. All p-values were considered significant at α-level = 0.05. All calculations were performed using the SAS V9.1 statistical software (SAS Institute Inc. Cary, NC).

RESULTS

Patient characteristics

Patients' characteristics are indicated in Table 1. The cohort included a total of 1102 patients, the majority of patients were male (86%) and the median age was 68 [60-74] years. Cirrhosis was present in 81% of patients; 73% of them were ranked Child-Pugh grade A. Underlying liver disease was related to alcohol in 41% of the patients, and to viral C hepatitis in 27% of the patients. HCC were multinodular in 59% of the cases and 43% of the patients had at least three nodules. Portal vein thrombosis was present in 41% of the cases, and 43% of HCCs were infiltrating tumors. Baseline ECOG performance status of our population (as expression of symptomatic tumor) was as follows: PS 0 (50%), PS 1-2 (46%), PS 3-4 (4%).

The stratification of patients according to the BCLC system was as follows: BCLC A (17%), BCLC B (16%), BCLC C (60%), and BCLC D (7%).

The primary anti-cancer treatments of patients are shown in Figure 1 and Table 1. twenty-three point five percent of the patients received treatments of curative intent (surgery, RFA ± TACE), while 59.5% of the patients received a palliative treatment (TACE, sorafenib, others systemic treatments) and 17% only best supportive care.

Survival analysis and stage-specific survival

Median overall survival for the entire cohort was 10.8 mo [4.9-28.0], consistent with the median follow-up duration: 10 mo [4.4-22.7]. Eighty-two percent of patients died. Median overall survival according to the BCLC system was as follows: BCLC A 43 mo [36-57],

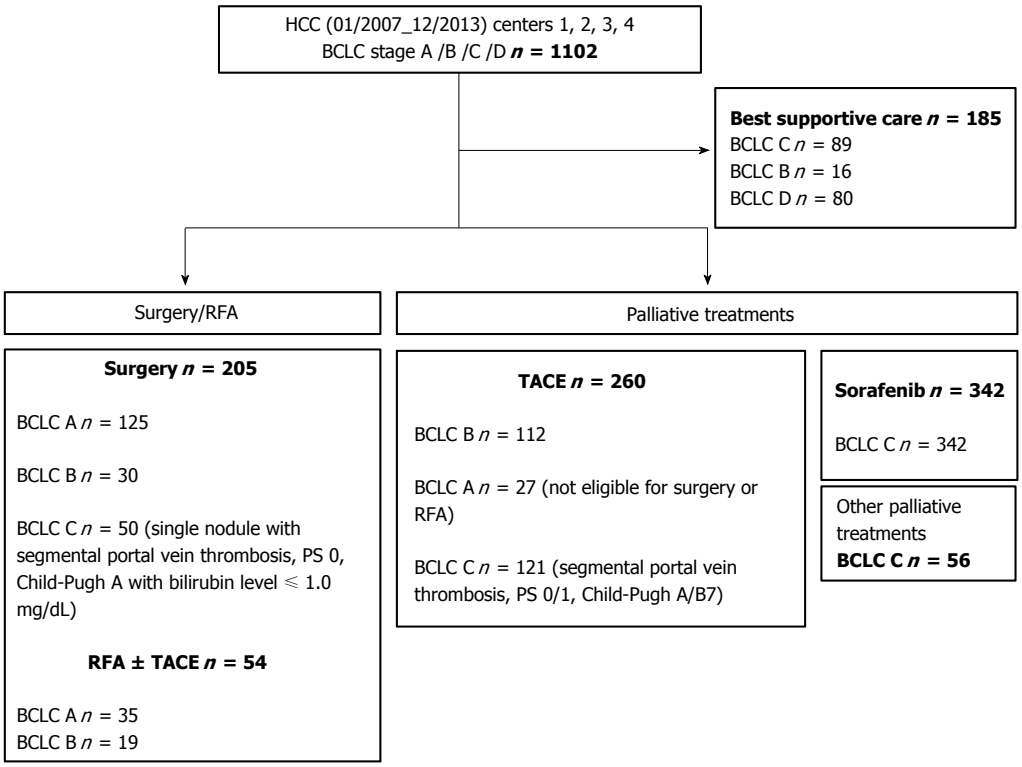

Figure 1 Flow diagram shows the patient selection criteria. BCLC: Barcelona Clinic Liver Cancer; HCC: Hepatocellular carcinoma; PS: Performance status; TACE: Trans arterial chemoembolization.

Table 1 Patients' characteristics at diagnosis (*n* = 1102) and first hepatocellular carcinoma recorded treatment *n* (%)

	All patients (*n* = 1102)
Age - Median (Q1-Q3), yr	68 (60-74)
Gender	
Male/Female	943 (86)/159 (14)
Liver disease	
Alcoholism/HCV/HBV/MS/Other	452 (41)/297 (27)/66 (6)/99 (9)/188 (17)
Cirrhosis	895 (81)
Child - Pugh grade	
A/B	653 (73)/242 (27)
Tumor Size (Q1-Q3) mm	43 (20-75)
Multifocal	654 (59)
Nodules	
< 3/⩾ 3	633 (57)/469 (43)
Portal vein thrombosis	452 (41)
Infiltrative HCC	469 (43)
AFP - Median [Q1-Q3], ng/mL	53 (7-1300)
ECOG (PS)	
0/1-2/3-4	553 (50)/506 (46)/43 (4)
BCLC stage	
A/B/C/D	187 (17)/177 (16)/658 (60)/80 (7)
Treatment allocation	
Resection/RFA ± TACE	259 (23.5)
TACE	260 (23.5)
Sorafenib	342 (31)
Other palliative treatments	56 (5)
Supportive care	185 (17)

HBV: Hepatitis B virus; HCV: Hepatitis C virus; MS: Metabolic syndrome; AFP: Alpha-foetoprotein; ECOG PS: Eastern Cooperative Oncology Group Performance Status; BCLC: Barcelona Clinic Liver Cancer; RFA: Radiofrequency ablation; TACE: Trans arterial chemoembolization.

BCLC B 19 mo [17-23], BCLC C 8 mo [7-9] and BCLC D 2 mo [2-3] (*P* (Log-Rank) < 0.0001) (Figure 2A).

The HKLC system differentiated within this cohort between nine subgroups with median overall survival ranging from 43 [36-55] mo for the HKLC group 1 to 3 [2-4] mo for the HKLC group 5b, *P* (Wilcoxon) < 0.0001. However, several subgroups (ⅡaⅡb, ⅢbⅣa, ⅣbⅤb) had a similar overall survival (Figure 3).

The CLIP and NIACE scores differentiated within this cohort seven and ten subgroups respectively with a different prognosis, *P* (Wilcoxon) < 0.0001 (Figure 3). CLIP scores ranked 74% of the patients in the first three groups (0 - 1 - 2): 19%, 30% and 25%, respectively. The distribution of patients in the ten subgroups from the NIACE score was more homogeneous (NIACE 0: 14%, 1: 8%, 1.5: 16%, 2.5: 11%, 3: 17%, 4: 12%, 4.5: 9%, 5.5: 8%, 6: 2% and NIACE 7: 3%).

The nomogram values within the cohort are shown in the Figure 4. In summary, the nomogram distinguished sixteen subgroups. Analysis of survival time based on nomogram BCLC values showed a significant difference, *P* (Wilcoxon) < 0.0001, survival time decreased with increasing nomogram values.

Comparison of predictive accuracy for overall survival between the nomogram and the conventional staging and scoring systems

Performances of the nomogram and other staging and scoring systems for survival prediction are indicated in Table 2. The C-index of the nomogram for predicting overall

Table 2 Comparison of predictive accuracy for overall survival between the nomogram and the conventional staging and scoring systems (Barcelona clinic liver cancer, HKLC, CLIP, NIACE)

Score	Discriminatory ability linear trend test		Homogeneity likelihood ratio test		Akaike information criterion	C-index
	LT (χ^2)	*P* value	LR (χ^2)	*P* value		
BCLC Nomogram	93.2169	< 0.0001	500.7218	< 0.0001	10679.513	0.719
NIACE	91.6906	< 0.0001	532.0369	< 0.0001	10648.198	0.718
BCLC	79.0342	< 0.0001	380.4100	< 0.0001	10805.825	0.674
HKLC	71.8861	< 0.0001	455.3169	< 0.0001	10740.918	0.698
CLIP	87.2785	< 0.0001	430.3872	< 0.0001	10749.848	0.716
Nomogram according to BCLC last version	86.1320	< 0.0001	417.4356	< 0.0001	10762.799	0.698

BCLC last version transfer single and large HCC > 50 mm from intermediate to early stages[3]. BCLC: Barcelona Clinic Liver Cancer; HCC: Hepatocellular carcinoma.

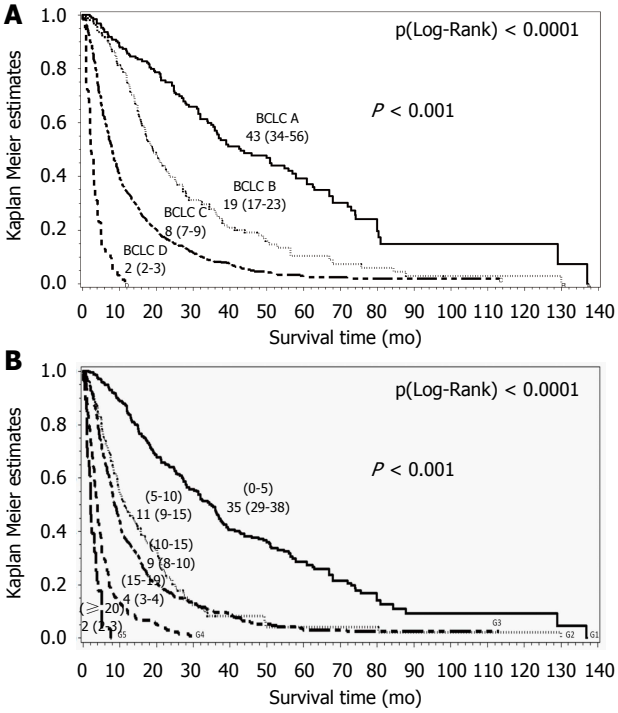

Figure 2 Kaplan-Meier estimated survival curves stratified according to Barcelona clinic liver cancer stages (A) or to Barcelona clinic liver cancer nomogram stratified in 5 classes (0-5), (5-10), (10-15), (15-19), (≥ 20) (B).

survival was 0.719, significantly higher than the BCLC system (0.674), the HKLC system (0.698). The nomogram yielded a higher discriminative ability (LT (χ^2) = 93.2169) than the other systems. The likelihood ratio test showed that the nomogram had an additional homogeneity of survival within each score (500.7218) close to the best value produced by the NIACE score (532.0369), and higher than other systems. Moreover, the nomogram was associated with a lower corrected Akaike information criterion (10679.513) compared with the other systems and close to the best value produced by the NIACE score (10648.198).

DISCUSSION

Our findings indicate that the nomogram has a good stratification ability with regard to prognosis in patients with HCC, within a European HCC cohort, mostly BCLC-C[47,48] compared to other known staging and scoring systems (BCLC, HKLC systems, CLIP score). By specifying the magnitude of each variable within the BCLC system (tumor burden, liver function, general conditions), the nomogram can better predict the survival of patients with HCC. In previous studies, CLIP and NIACE scores showed a better predictive value for survival compared to other staging and scoring systems within two large Asian and European HCC cohorts[15,25,26].

In our study the CLIP score also distinguished between subgroups with significantly different survival, but the majority of patients (74%) were in the first three groups (CLIP 0, 1 and 2), as previously described[15,49,50], limiting its discriminatory capacity.

The HKLC classification proposes another stratification with five groups and nine subgroups in order to enhance prognostic accuracy for HCC; the early stages (Ⅰ, Ⅱa) include BCLC A and B HCC patients, the intermediate stages (Ⅱb, Ⅲa) include BCLC A, B and C HCC patients and the locally advanced stages (Ⅲb) include BCLC B and C HCC patients. Despite a greater number of subgroups, some of them had the same survival (Ⅱa/Ⅱb, Ⅲb/Ⅳa and Ⅳb/Ⅴb), as previously reported[51], reducing the usefulness of this new classification in a European cohort.

The nomogram showed a higher predictive power for survival within this external European cohort, but there is still some issue. The nomogram is a reliable predictor of survival for patients with HCC, however this nomogram is complex ranging from 0 to 26 points and in our cohort, it distinguished sixteen subgroups. Moreover, it doesn't help clinicians in treatment decision. A simplified stratification into five sub-groups is possible: [0-5], [5-10], [10-15], [15-19], and [≥ 20]; the survival time observed in our cohort was respectively: 35 [30-38] mo, 12 [10-16] mo, 9 [8-10] mo, 4 [3-4] mo, and 2 [2-3] mo, *P* < 0.0001 (Figure 2B). These results should be validated, or other thresholds may be suggested by a specific analysis.

There is another issue with the nomogram after

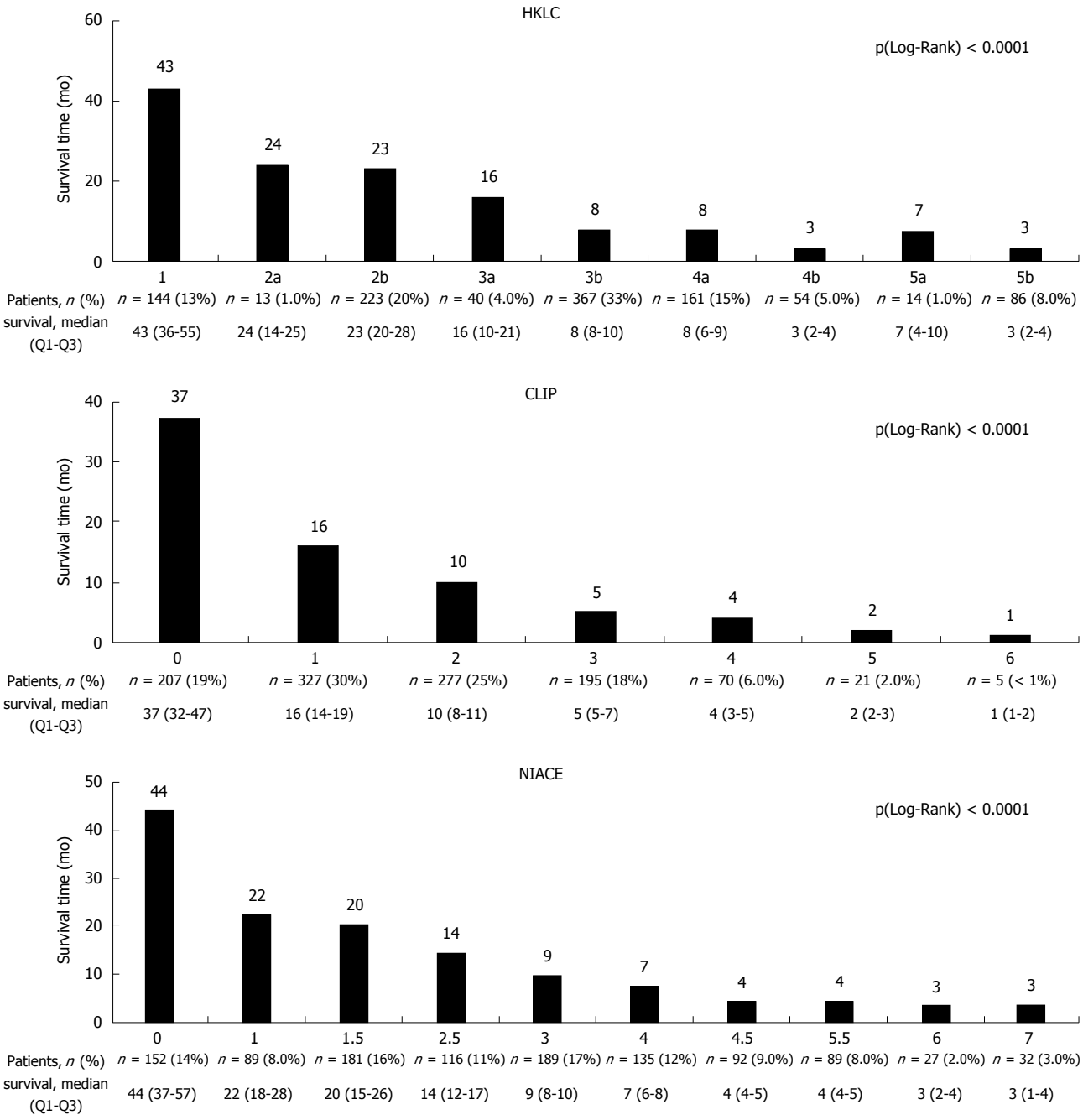

Figure 3 Overall survival Histograms according to HKLC staging system, CLIP score and NIACE score in our hepatocellular carcinoma cohort.

the adoption of changes in the BCLC system[3], which could affect its discriminatory capacity. Single and large tumors (> 50 mm) were included into the BCLC A group; therefore, they should logically be included in the tumor burden grade 1 and not 2. By applying this rule, the predictive value of the nomogram became lower (c-index: 0.698 *vs* 0.719) (Table 2).

In addition, the prognostic accuracy of the nomogram and the NIACE are close within this cohort. However, NIACE score is not only an additional prognostic score to the BCLC system[22,26], but it can be used as an aid to the decision-making process, distinguishing different prognostic groups among patients treated by surgery or those treated by TACE or Sorafenib[22,24]. The combination of classification plus scores (BCLC and NIACE) have already showed an additional value for treatment recommendation in a retrospective cohort and prospective validation study should be designed[52].

There are several limitations of the present study including the retrospective study design, its multicenter nature, which may make bias unavoidable. Regarding treatment decision, BCLC treatment recommendations are seldom followed due to great heterogeneity within each stage[48,53,54]. In our study, 33% of patients received treatment outside BCLC recommendations [14% of BCLC A HCC patients (*n* = 27), 28% of BCLC B HCC patients (*n* = 49), and 40% of BCLC C HCC

Performance Status (PS) 3-4: 6.7 points, PS 1-2: 3 points
Child-Pugh-Turcotte CPT C: 8.9 points, CPT B: 5.2 points
Tumor burden (TB) grade 3: 10 points, TB grade 2: 3.7 points, TB grade 1: 1.2 points

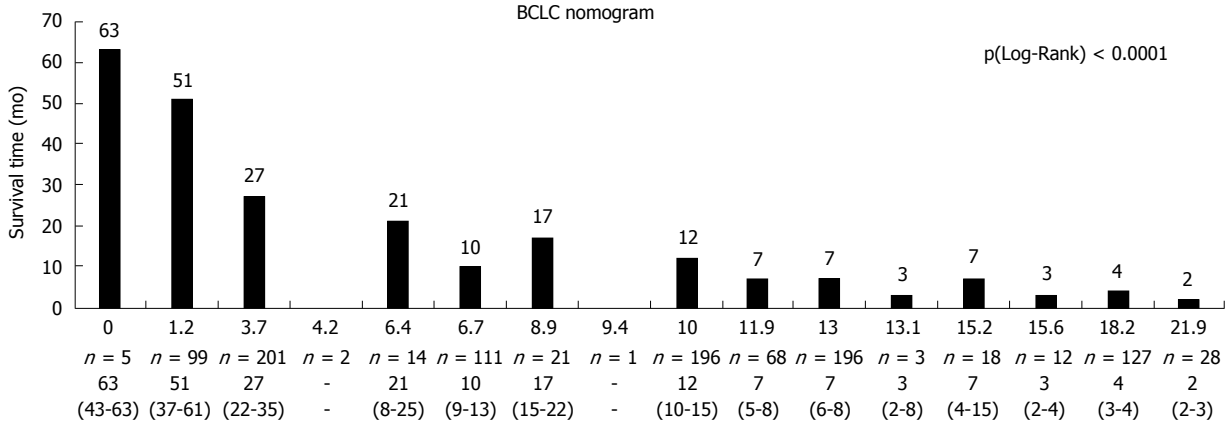

Figure 4 Survival time in months according to hepatocellular carcinoma nomogram. Hsu *et al*[27] in our hepatocellular carcinoma cohort.

patients (n = 227)]. sixty-two percent of patients undergoing surgery or RFA were ranked as BCLC A HCC, 43% of patients treated by TACE were ranked as BCLC B HCC, and 40% of treated BCLC C patients received a first-line treatment other than sorafenib. Our cohort mainly included advanced HCC, that is a heterogeneous population with limited therapeutic option until now, namely sorafenib with modest survival benefit[55] or inclusion in randomized trials who do not reflect patients in daily clinical practice. In our study like others[56-59] impairment of liver function is the major factors that preclude patient to receive sorafenib. Moreover BCLC-C patients before sorafenib availability have received others non-valuable treatment. Each BCLC stage including a broad spectrum of tumors, a proportion of patients in each stage do not fulfill all the criteria for the treatment allocation, and for some authors other therapeutic options are possible[8,54,60,61]. Therefore treatment recommendations based on new combination of BCLC and scoring systems such as NIACE or other are urgently required.

In summary, this study confirms the BCLC nomogram as a new HCC reliable prognostic tool; its predictive value on survival is higher compared to known classifications and scoring system. However, the usefulness of this nomogram is limited due to its complexity and the fact that it is not linked to a therapeutic strategy. BCLC system remains the most widely used staging system, however BCLC treatment recommendations are seldom followed suggesting the need for better tools.

COMMENTS

Background

Hepatocellular carcinoma (HCC) prognosis is still a controversial issue. Barcelona Clinic Liver Cancer staging system has limits [heterogeneity of the Barcelona clinic liver cancer (BCLC) subgroups, strict therapeutic algorithm]. Using a nomogram as proposed by Hsu *et al* to improve BCLC system prognostic value is an attractive idea for clinicians.

Research frontiers

Hsu *et al* think that conferring value on each of the three main parameters of the BCLC system ie tumor burden, liver function and performance status (using a multivariate Cox regression model within a large Asian HCC cohort), could improve the individual prognosis of HCC patients. The authors think that prognosis and treatment of HCC should be associated. They assessed the reliability and the usefulness of the BCLC nomogram within a European cohort mainly related to alcohol abuse and HCV hepatitis.

Innovations and breakthroughs

This paper shows that the BCLC nomogram is a reliable tool for HCC prognosis, irrespective of the underlying liver disease, with a better predictive value for survival compared to other scoring or staging systems (CLIP, HKLC).

But its usefulness is limited by its complexity (tumor burden grade 3: 10 points, grade 2 and 1: 3.7 and 1.2 points; Child-Pugh grade C: 8.9 points, Child-Pugh grade B and A: 5.2 and 0 points; PS 3-4: 6.7 points, PS 1-2 and 0: 3 and 0 points) and the lack of therapeutic link. They Suggest an additional score (including other prognostic variables such as AFP serum level and/or tumor morphology) to the BCLC system in order to improve the prognostic information and the therapeutic decision.

Applications

BCLC nomogram provides reliable prognostic information for HCC patients, irrespective of underlying liver disease, but it doesn't guide the therapeutic decision. Conversely a combination of BCLC system and scores may influence HCC prognosis and its therapeutic management.

Terminology

NIACE score (tumor Nodularity, Infiltrative nature of the tumor, serum Alpha-fetoprotein level, Child-Pugh stage, ECOG performance status) determines sub-groups of different survival prognosis irrespective of the BCLC stage, or HCC treatment modalities.

Peer-review

The aim of this study is to compare the performances of several HCC staging systems including the BCLC nomogram in the prediction of survival of a large French HCC cohort. A total of 1102 HCC patients retrospectively recruited from 5 hospitals in different areas. The objective of this study is clear and the statistical studies were well done. The conclusion is logical and adequate.

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ORIGINAL ARTICLE

Retrospective Study

Laparoscopic approach to suspected T1 and T2 gallbladder carcinoma

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Author contributions: Ome Y designed the study, gathered the clinical data, and wrote the manuscript; all authors decided on courses of treatment and performed the operations; Ome Y, Hashida K, Yokota M and Nagahisa Y analyzed the data; all authors helped with drafts, reviewed the manuscript, and approved it.

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Abstract

AIM

To evaluate a laparoscopic approach to gallbladder lesions including polyps, wall-thickening lesions, and suspected T1 and T2 gallbladder cancer (GBC).

METHODS

We performed 50 cases of laparoscopic whole-layer cholecystectomy (LCWL) and 13 cases of laparoscopic gallbladder bed resection (LCGB) for those gallbladder lesions from April 2010 to November 2016. We analyzed the short-term and long-term results of our laparoscopic approach.

RESULTS

The median operation time was 108 min for LCWL and 211 min for LCGB. The median blood loss was minimal for LCWL and 28 ml for LCGB. No severe morbidity occurred in either procedure. Nine patients who underwent LCWL and 7 who underwent LCGB were postoperatively diagnosed with GBC. One of these patients had undergone LCGB for pathologically diagnosed T2 GBC after LCWL. All of the final surgical margins were negative. Three of these 15 patients underwent additional open surgery. The mean follow-up period was 26 mo, and only one patient developed recurrence.

CONCLUSION
LCWL and LCGB are safe and useful procedures that allow complete resection of highly suspected or early-stage cancer and achieve good short-term and long-term results.

Key words: Laparoscopic cholecystectomy; Whole-layer cholecystectomy; Gallbladder bed resection; Radical cholecystectomy; Gallbladder carcinoma

Core tip: Laparoscopic cholecystectomy is commonly performed for the treatment of benign diseases. Gallbladder carcinoma (GBC) is typically managed by open surgery because of various concerns associated with potential dissemination, recurrence, and technical difficulties. However, many benign lesions are difficult to differentiate from GBC, including polyps and lesions that cause wall thickening. We use a laparoscopic approach for many types of gallbladder lesions including gallbladder carcinoma. This study demonstrated that our laparoscopic approach is safe, useful, and allows for the complete resection of highly suspected or early-stage gallbladder cancer.

Ome Y, Hashida K, Yokota M, Nagahisa Y, Okabe M, Kawamoto K. Laparoscopic approach to suspected T1 and T2 gallbladder carcinoma. *World J Gastroenterol* 2017; 23(14): 2556-2565 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i14/2556.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i14.2556

INTRODUCTION

Laparoscopic cholecystectomy (LC) is a basic approach for benign diseases such as cholecystolithiasis. However, laparoscopic surgery for gallbladder carcinoma (GBC) has not been widely employed. This is because of the highly malignant potential of GBC, higher rates of port-site recurrence (PSR) and peritoneal dissemination caused by intraoperative perforation of the gallbladder in LC than in open cholecystectomy, and the technical difficulties involved in the laparoscopic performance of standard GBC procedures[1]. Meanwhile, many benign lesions are difficult to differentiate from GBC, including polyps and wall thickening lesions such as chronic cholecystitis and adenomyomatosis[2-12]. Such lesions are sometimes diagnosed as GBC postoperatively by pathological examination. Achieving the correct preoperative diagnosis and stage of GBC is very difficult[13]. Tumor exposure as well as intraoperative gallbladder perforation can increase the risk of cancer relapse; therefore, the above-mentioned lesions, which are associated with suspected GBC, should be carefully managed. We use a laparoscopic approach depending on the type of gallbladder lesion, including suspected T1 and T2 GBC, in our institution. In this study, we evaluated the short-term and long-term outcomes of our strategy.

MATERIALS AND METHODS

Laparoscopic approach

Since April 2010, we have used a laparoscopic approach depending on the type of gallbladder lesion being treated. A laparoscopic approach is indicated for polyps larger than 10 mm, growing polyps, wall-thickening lesions including chronic cholecystitis and adenomyomatosis, and suspected T1 or T2 GBC. Computed tomography, magnetic resonance imaging, and abdominal ultrasonography are routinely carried out as preoperative examinations. When GBC is highly suspected and a more exact differential diagnosis is required, clinicians may consider the use of endoscopic ultrasonography, positron emission tomography, and multidetector computed tomography. An algorithm of our laparoscopic approach to gallbladder lesions is shown in Figure 1. Intraoperative ultrasonography (IOUS) is usually performed first during the operation. We perform laparoscopic whole-layer cholecystectomy (LCWL) for suspected benign lesions rather than for GBC. In conventional cholecystectomy, the gallbladder is dissected along the inner layer of the subserosal layer[14]. On the other hand, the gallbladder is removed including the cystic plate by dissecting along the outer layer of the subserosal layer in whole-layer cholecystectomy. These benign lesions include wall-thickening lesions such as adenomyomatosis and chronic cholecystitis, pedunculated polyps on the peritoneal side or smaller than 15 mm, and sessile polyps on the peritoneal side and smaller than 15 mm. When intraoperative or postoperative pathological examination unexpectedly reveals the presence of GBC invading beyond the muscular layer, additional gallbladder bed resection and regional lymphadenectomy are considered. These additional procedures were previously performed by open surgery but can now be performed laparoscopically because of technical improvements. However, we perform laparoscopic gallbladder bed resection (LCGB) for pedunculated polyps on the liver side and larger than 15 mm, sessile polyps on the liver side or larger than 15 mm, and suspected T1 and T2 GBC. In Japan, D1 lymphadenectomy is defined as removal of the lymph nodes around the cystic duct and common bile duct, and D2 lymphadenectomy is defined as removal of the lymph nodes in the hepatoduodenal ligament, around the common hepatic artery, and around the posterosuperior region of the pancreas head. We perform LCGB with D2 lymphadenectomy for strongly suspected or definite T2 GBC, but with D1 lymphadenectomy for other lesions.

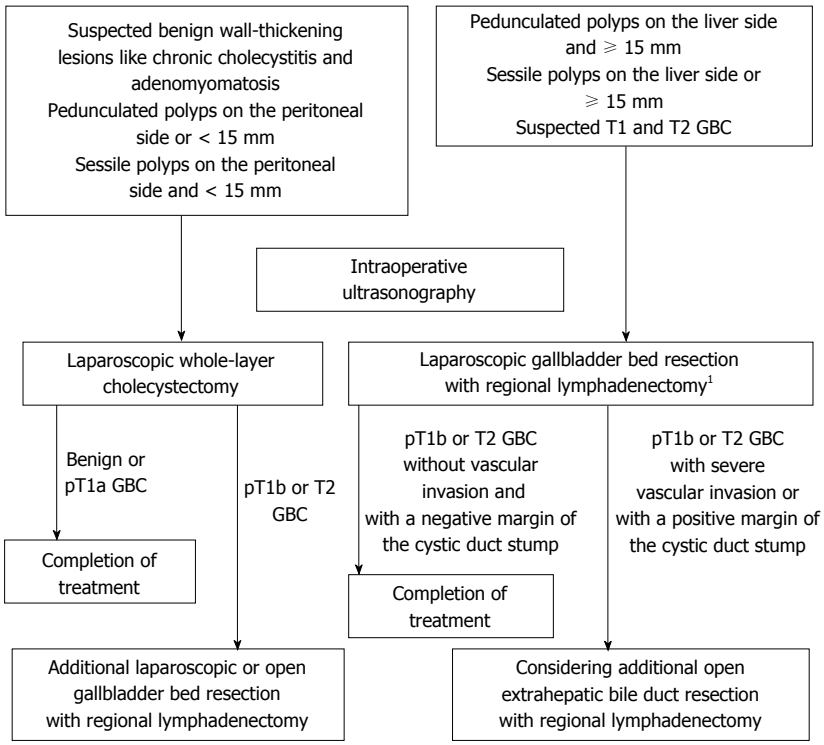

Figure 1 Algorithm of our laparoscopic approach to gallbladder lesions. [1]D2 lymphadenectomy for suspected T2 GBC, and D1 lymphadenectomy for the others. D1 lymphadenectomy is defined as removal of the lymph nodes around the cystic duct and the common bile duct. D2 lymphadenectomy is defined as removal of the lymph nodes in hepatoduodenal ligament, around the common hepatic artery, and around the posterosuperior region of the pancreas head. GBC: Gallbladder carcinoma.

Laparoscopic whole-layer cholecystectomy

The trocars in LCWL are positioned as in conventional LC. A 12-mm trocar for the laparoscope is placed on the umbilicus, a 5-mm trocar in the epigastric region, and two 5-mm trocars in the right subcostal area. Alternatively, LCWL can be performed by a single-incision approach (Figure 2A). First, IOUS is performed to examine the lesion and investigate the extent of the tumor. The cystic duct and the cystic artery are separated and cut, and the sentinel lymph nodes (around the cystic duct) are removed to check for lymph node metastasis. When the tumor extension approaches the cystic duct, the cystic artery and duct are cut at their origin. We take special care to avoid grasping the tumor site and causing perforation during the cholecystectomy. The adipose tissues and cystic plate of Calot's triangle are resected. The surface of the liver parenchyma, which is covered by a glossy membrane called Laennec's capsule, is then exposed. The whole-layer gallbladder wall, which includes the cystic plate, is easily detached from the liver bed by blunt dissection without bleeding, leaving Laennec's capsule on the liver surface (Figure 2B and C). A drain is usually unnecessary. The resected specimen is inserted into a retrieval bag and extracted though the umbilical port site.

Laparoscopic gallbladder bed resection

A 12-mm trocar for the laparoscope is usually placed on the umbilicus, a 12-mm trocar is placed in the epigastric region, and two 5-mm trocars are placed in the right subcostal area. When we plan to perform D2 regional lymphadenectomy, a 12-mm trocar for the laparoscope is also inserted through the umbilicus, a 12-mm trocar is inserted into the right flank region, a 5-mm trocar is inserted into the left flank region, and two 5-mm trocars are inserted into the right and left subcostal areas, respectively (Figure 3A and B). First, IOUS is performed as described above. We sometimes cut and retract the round ligament to maintain a good operative field. In D1 lymphadenectomy, the hepatoduodenal ligament above the upper margin of the pancreas is dissected using laparoscopic coagulating shears (LCS). The right hepatic artery and cystic artery are identified, and the cystic artery is cut at its origin. The cystic duct is clamped and cut at its origin, and the lymph nodes are removed from the common bile duct and right hepatic artery (Figure 3C). We do not completely remove the lymph nodes in the hepatoduodenal ligament during the first operation when the presence of GBC and the depth of its invasion are uncertain; however, we intend to resect the lymph nodes around the cystic artery, cystic duct, and common bile duct, including the sentinel lymph nodes, and to achieve a negative surgical margin. In D2 lymphadenectomy for suspected or definite T2 GBC, Kocher's mobilization is fully performed and the inferior vena cava and left renal vein are then

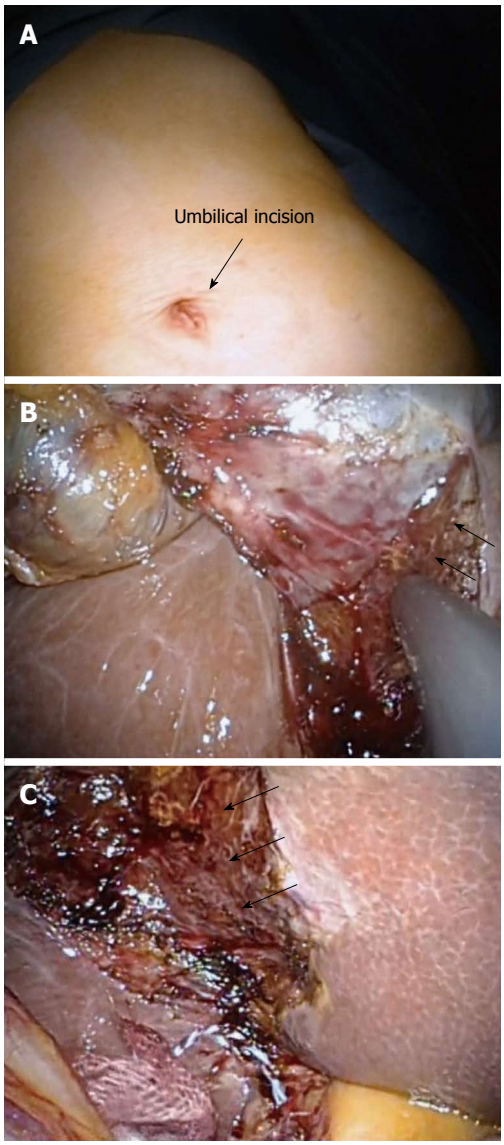

Figure 2 Surgical procedure for laparoscopic whole-layer cholecystectomy. A: The wound just after single-incision laparoscopic whole-layer cholecystectomy; B: Detachment of the whole-layer gallbladder wall from the liver bed, leaving Laennec's capsule (arrow) on the liver surface; C: After resection of the gallbladder.

identified (Figure 3D). Next, the lymph nodes around the posterosuperior region of the pancreas head are removed (Figure 3E). The magnified laparoscopic view enables us to accurately identify the boundary between the pancreatic parenchyma and surrounding adipose tissues to allow for safe dissection of the lymph nodes from the pancreas. The vessels around the pancreas, such as the posterior superior pancreaticoduodenal artery and vein, are effective guides for the dissection, and the dissection proceeds along those vessels. Lymphadenectomy is continued along the superior border of the pancreas and common hepatic artery. The lymph nodes are then dissected along the portal vein, proper hepatic artery, left and right hepatic arteries, and common bile duct. We are careful to avoid excessive exposure of the common bile duct and to preserve the pericholedochal vessels, thus avoiding delayed biliary stenosis. The cystic artery and duct are clamped and cut at their origin, and regional lymphadenectomy is completed (Figure 3F). The Pringle maneuver is usually performed with an extracorporeal tourniquet during liver parenchymal transection (Figure 3G). The resection line of the liver is determined about 1 to 2 cm away from the gallbladder bed margin. The superficial layer of the resection area is dissected using LCS. The liver parenchyma is transected by the clamp crushing method using LCS or a bipolar device, and the remaining fibrous tissues and small vessels are cut using LCS (Figure 3H). A comparatively large vein should be carefully separated, clamped, and cut. After resection of the gallbladder bed has been completed, careful hemostasis is finally confirmed (Figure 3I). A drain is placed through the foramen of Winslow. The resected specimen is inserted into a retrieval bag and extracted though the enlarged umbilical port site.

Patients

From April 2010 to November 2016, 52 patients underwent LCWL and 13 underwent LCGB for gallbladder lesions suspected to be GBC. Two of the 52 patients who underwent LCWL with simultaneous resection of another cancer site were excluded from this study. The patient characteristics, perioperative findings, pathological findings, and postoperative outcomes of the patients who underwent LCWL or LCGB were retrospectively reviewed, and the short-term and long-term outcomes of our laparoscopic approach were analyzed.

Statistical analysis

The patients' characteristics are expressed as median with range for continuous data and as number with percentage for categorical data. The RFS rates were estimated using the Kaplan-Meier method. These analyses were performed using SPSS, version 20.0 (IBM Corp., Armonk, NY, United States).

RESULTS

The patients' perioperative characteristics, including their clinicopathological and surgical data, are summarized in Table 1. There were no conversions from either LCWL or LCGB to open surgery. The median operation time was 108 (61-221) min for LCWL and 211 (111-269) min for LCGB. The median blood loss was minimal (> 0-150) mL for LCWL and 28 (> 0-150) mL for LCGB. Intraoperative perforation was seen in only one patient (2.0%) who underwent LCWL. There were no instances of severe postoperative complications (Clavien-Dindo grade $\geqslant$ 3) and no mortality in either procedure. The length of the postoperative hospital stay was 3 (1-6) d for LCWL and 6 (4-11) d for LCGB. The data of the patients pathologically diagnosed

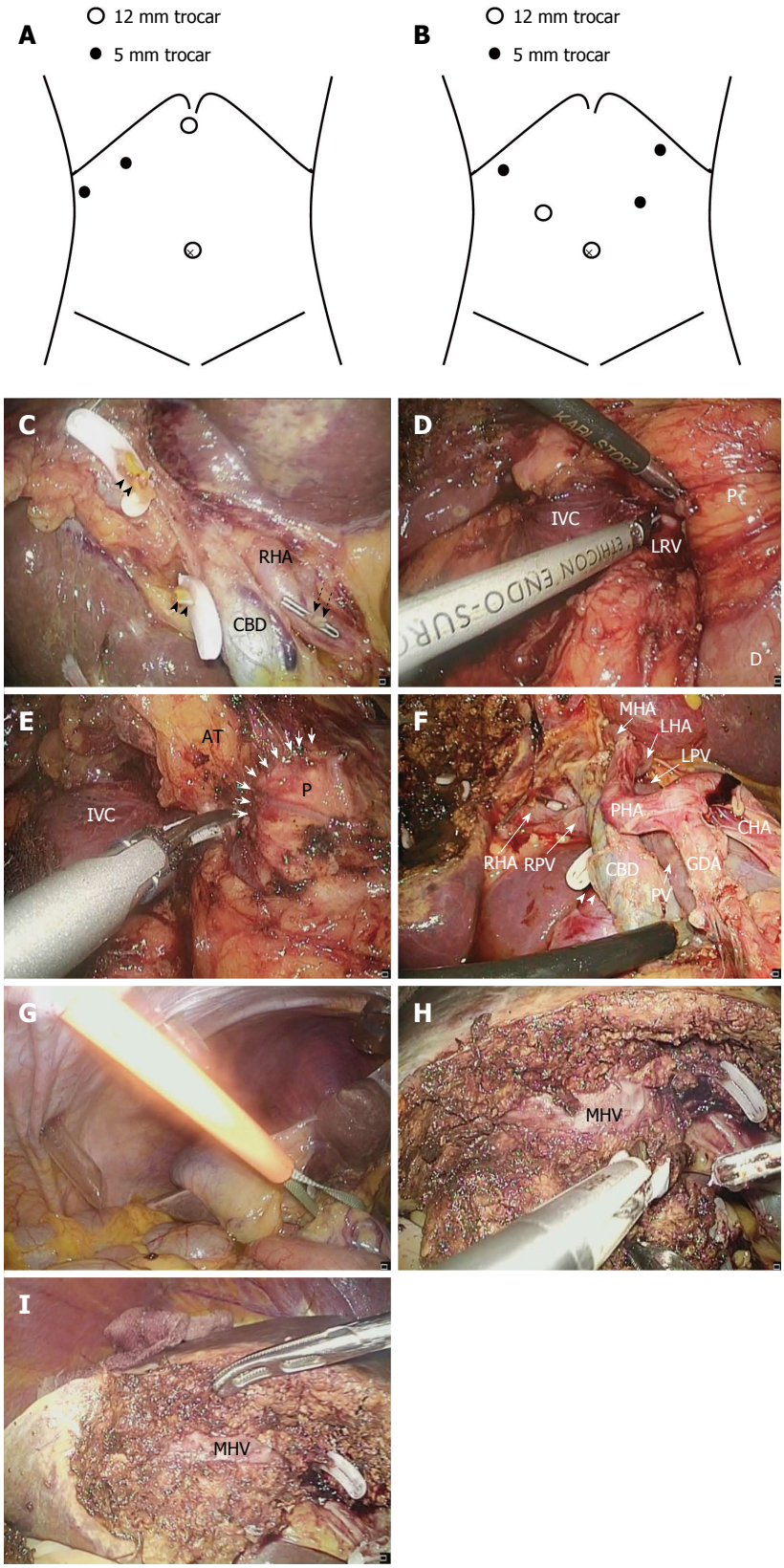

Figure 3 Surgical procedure for laparoscopic gallbladder bed resection. A: Position of trocars in laparoscopic gallbladder bed resection (LCGB) with D1 lymphadenectomy; B: Position of trocars in LCGB with D2 lymphadenectomy; C: The cystic artery and duct are cut at their origin; D: Kocher's mobilization; E: Lymph node dissection around the posterosuperior region of the pancreas head. Arrow indicates the boundary between the pancreatic parenchyma and surrounding adipose tissues; F: Completion of D2 lymphadenectomy; G: Performance of the Pringle maneuver with an extracorporeal tourniquet; H: Transection of the liver parenchyma by the clamp crushing method; I: After the gallbladder bed resection. RHA: Right hepatic artery; CBD: Common bile duct; Arrowhead: Stump of the cystic duct; Dotted arrow: Stump of the cystic artery; P: Pancreatic head; D: Duodenum; IVC: Inferior vena cava; LRV: Left renal vein; AT: Adipose tissues; GDA: Gastroduodenal artery; CHA: Common hepatic artery; PHA: Proper hepatic artery; LHA: Left hepatic artery; MHA: Middle hepatic artery; PV: Portal vein; LPV: Left portal vein; RPV: Right portal vein; MHV: Middle hepatic vein.

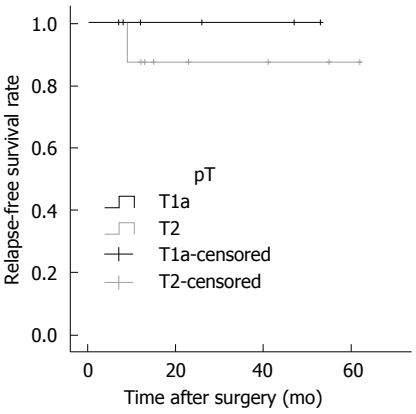

Figure 4 Relapse-free survival rate after laparoscopic surgery for pathologically diagnosed T1a and T2 gallbladder carcinoma.

with GBC are shown in Table 2. Among patients who underwent LCWL, nine were postoperatively diagnosed with GBC. The depth of invasion was pT1a in four patients, pT2 in four patients, and pT3 in one patient. Only the one patient with pT3 invasion had lymph node metastases. Two patients with pT2 GBC and one patient with pT3 GBC underwent additional resection. We performed open S4a and S5 segmentectomy with extrahepatic bile duct resection (EBR) and regional lymphadenectomy in one patient with pT2 and one patient with pT3 GBC. One patient (Case No. 9) with pT2 GBC underwent LCGB with D2 lymphadenectomy without EBR as additional resection. The other two patients with pT2 GBC did not undergo additional resection because of their old age. On the other hand, among the patients who underwent LCGB, seven were diagnosed with GBC. One of them was the above-mentioned patient (Case No. 9) who had previously undergone LCWL. The depth of invasion in patients who underwent LCGB was pT1a in two patients and pT2 in five patients. Two of the five patients with pT2 GBC had lymph node metastases. We performed LCGB with D2 lymphadenectomy for three patients with pT2 GBC, including the above-mentioned patient (Case No. 9). One patient with pT2 GBC underwent additional open lymphadenectomy with EBR. In both LCWL and LCGB, all of the final surgical margins were pathologically negative. The mean follow-up period after the operation for GBC was 26 mo, and only one patient (6.7%) who had undergone LCGB with D2 lymphadenectomy for pT2 GBC with multiple lymph node metastases (Case No. 7) developed recurrence 9 mo after the operation and died 14 mo postoperatively. One patient (Case No. 3) died 2 mo after LCWL and 1 mo after the additional resection because of another disease that was not associated with the surgical procedure or the GBC. No port site recurrence or peritoneal dissemination was found. The postoperative RFS rate in patients who underwent the laparoscopic approach is shown in Figure 4.

Table 1 Perioperative characteristics of the patients *n* (%)

	LCWL (*n* = 50)	LCGB (*n* = 13)
Clinical findings		
Sex		
Male	30 (60)	6 (46.2)
Female	20 (40)	7 (53.8)
Age, median (range, yr)	58.5 (30-92)	67 (50-85)
Surgical findings		
Operation time, median (range, min)	108 (61-221)	211 (111-293)
Intraoperative blood loss, median (range, mL)	Minimal (> 0-150)	28 (> 0-150)
Intraoperative perforation	1 (2.0)	0 (0)
Conversions to the open approach	0 (0)	0 (0)
Perioperative outcomes		
Severe postoperative complications[1]	0 (0)	0 (0)
Mortality	0 (0)	0 (0)
Postoperative hospital stay, days -median (range)	3 (1-6)	6 (4-11)
Pathological findings		
Gallbladder carcinoma	9 (18)	7 (53.8)
Depth of invasion[2]		
pT1a	3	2
pT1b	0	0
pT2	5	5[3]
pT3	1	0
pT4	0	0
Lymph node metastasis		
pN0	9	5
pN1	0	2
Surgical margin		
positive	0	0
negative	9	7
Postoperative outcomes		
Additional operation performed	3	1
Recurrence		
Yes	0	1
No	9	6

[1]Clavien-Dindo grade ⩾ 3 complications; [2]Tumors were classified according to the American Joint Committee on Cancer (AJCC)/TNM system; [3]One patient underwent LCGB with D2 lymphadenectomy 42 d after LCWL. LCWL: Laparoscopic whole-layer cholecystectomy; LCGB: Laparoscopic gallbladder bed resection.

DISCUSSION

LC is a common procedure for treatment of benign disease. Several studies on laparoscopic radical resection for GBC have also been reported[15-18]. However, the performance of laparoscopic surgery for GBC has not become widespread. The reasons for this are associated with the highly malignant potential of GBC and technical difficulties in performing regional lymphadenectomy and gallbladder bed resection.

The dissection during conventional LC on the liver side is performed along the inner layer of the subserosal layer. When the depth of invasion of the GBC extends to the subserosal layer, residual GBC may exist after conventional LC. Even a mucosal carcinoma in the Rokitansky-Aschoff sinus may result in a positive surgical margin. Moreover, LC for GBC has serious problems with respect to PSR and peritoneal

Table 2 Data of patients pathologically diagnosed with gallbladder cancer

Case	Sex	Age	Preoperative diagnosis	Type of operation	pT	pN	pSM	Additional surgery	Adjuvant therapy	Recurrence	RFS (mo)	Outcome
1	F	71	polyp	LCWL	T2	N0	Negative	S4a and S5 segmentectomy with extrahepatic bile duct resection	No	No	62	Alive
2	F	80	polyp	LCWL	T1a	N0	Negative	-	No	No	53	Alive
3	M	79	chronic cholecystitic	LCWL	T3	N1	Negative	S4a and S5 segmentectomy with extrahepatic bile duct resection	No	No	2	Dead
4	F	80	GBC	LCGB	T2	N0	Negative	Extrahepatic bile duct resection	No	No	55	Alive
5	F	80	GBC	LCGB	T1a	N0	Negative	-	No	No	47	Alive
6	F	83	polyp	LCWL	T2	N0	Negative	-	No	No	41	Alive
7	F	61	GBC	LCGB	T2	N1	Negative	-	No	Liver and bone metastases	9	Dead
8	F	85	GBC	LCGB	T1a	N0	Negative	-	No	No	26	Alive
9	F	66	polyp	LCWL	T2	N0	Negative	LCGB with lymphadenectomy	No	No	23	Alive
9	F	66	definite GBC	LCGB	T2	N0	Negative	-	No	No	-	Alive
10	M	83	GBC	LCGB	T2	N0	Negative	-	No	No	15	Alive
11	M	84	polyp	LCWL	T2	N0	Negative	-	No	No	13	Alive
12	F	78	GBC	LCGB	T2	N1	Negative	-	No	No	12	Alive
13	M	50	polyp	LCWL	T1a	N0	Negative	-	No	No	12	Alive
14	F	86	GBC	LCWL	T1a	N0	Negative	-	No	No	8	Alive
15	F	92	polyp	LCWL	T1a	N0	Negative	-	No	No	7	Alive

LCWL: Laparoscopic whole-layer cholecystectomy; LCGB: Laparoscopic gallbladder bed resection; pSM: Pathological surgical margin; RFS: Relapse-free survival; GBC: Gallbladder cancer.

dissemination because of the bile leakage caused by intraoperative perforation. The incidence of PSR after LC for GBC has been reported to range from 11% to 16%[19-22]. Ouchi *et al*[23] reported that gallbladder perforation occurred in 20% of patients with GBC who underwent LC. Wakai *et al*[19] reported that gallbladder injury occurred in 25% of patients, of whom PSR or local recurrence developed in 43% who underwent LC among 28 patients with GBC. Both research groups found that patients with gallbladder perforation had a significantly poorer prognosis than did those without perforation. Lee *et al*[24] described that the incidence of PSR and peritoneal dissemination in patients who underwent LC was higher than that in patients who underwent open cholecystectomy. As just described, laparoscopic surgery for GBC is associated with several difficulties. However, laparoscopic surgery has been widely employed for other various malignancies, and it provides patients with a minimally invasive treatment and early recovery from the surgery. If we can overcome the defects of laparoscopic surgery for GBC, such patients will benefit from this procedure.

GBC is sometimes encountered incidentally. Accurate preoperative diagnosis of GBC and its depth of invasion is difficult. In particular, some lesions, such as chronic cholecystitis (the prime example is xanthogranulomatous cholecystitis) and adenomyomatosis, are important differential diagnoses of GBC that may be difficult to confirm[3-7]. Several studies have reported that polypoid lesions are highly suspected to be GBC when they are larger than 10 mm, solitary, sessile, or rapidly growing[8-12]. Yeh *et al*[2] reported that polypoid gallbladder lesions larger than 15 mm are more strongly suspected to be malignancies. The above-mentioned lesions should be more carefully treated to avoid causing bile spillage and tumor exposure.

We now use the laparoscopic approach on the types of gallbladder lesions shown in Figure 1. The present study has shown that the risk of gallbladder perforation in both LCWL and LCGB was much lower (2.0% and 0.0%, respectively) than that in conventional LC because the dissection layer is more outside than usual. The entire subserosal layer is removed by LCWL; thus, T1 or T2 GBC can be completely resected in theory. However, suspected GBC is now indicated for LCGB so that a safety margin from the tumor is secured. Proper indications for LCWL and LCGB help to avoid tumor exposure and achieve complete resection of GBC. Intraoperative pathological examination is not always performed because of our institutional system. The specimen is macroscopically evaluated immediately after its extraction to confirm the existence of GBC and negative surgical margins. Further investigation by pathological examination is performed postoperatively. We believe that it is most important to achieve complete resection of the tumor and an accurate diagnosis, including staging, without increasing the risk of recurrence in the first-stage operation. Therefore, we take extreme care to avoid tumor exposure and bile spillage during the first-stage

operation; if necessary, we plan an additional second-stage surgery.

When the diagnosis of GBC is made by intraoperative or postoperative pathological examination, an additional resection including regional lymphadenectomy should be considered depending on the depth of GBC invasion. In patients diagnosed with T1a carcinoma, an additional resection is not necessary if the surgical margin of the cystic duct stump is negative. However, an additional extended radical resection including regional lymphadenectomy is recommended in patients with T1b or more advanced GBC because vascular and perineural invasion and positive lymph node metastasis are observed at high rates[25-29]. Such an additional resection was previously performed by open surgery. However, now it can be carried out laparoscopically because of technical improvements. The laparoscopic magnified view allows for more accurate identification of the dissection plane and performance of finer procedures than in open surgery. These are great advantages of laparoscopic surgery, and can lead to reduced bleeding volume and accurate lymph node dissection.

The necessity of routine EBR is controversial[25,30-33]. There is currently no obvious evidence that recommends the routine EBR[1]. We usually perform regional lymphadenectomy without EBR when ductal involvement is not present. The advantages of EBR are facilitation of regional lymphadenectomy, removal of the possible presence of microscopic periductal involvement, and avoidance of postoperative ischemic biliary stenosis. The laparoscopic magnified view allows for sufficient lymph node dissection around the common bile duct and preservation of pericholedochal small vessels to prevent biliary ischemia. However, the lymphatic infiltration around the bile duct is a main pathway for tumor spread[25,34]. Therefore, when positive lymph node metastasis or advanced microscopic neurovascular invasion is detected, we now perform thorough regional lymphadenectomy with EBR by laparotomy, not by laparoscopic surgery.

Several studies have revealed that the long-term survival of patients with T1 or T2 GBC treated by laparoscopic radical cholecystectomy was comparable with that of patients treated by open surgery[17,18]. In the present study, our laparoscopic approach achieved good short-term outcomes and provided acceptable long-term outcomes although there was a limitation that the follow-up period was comparatively short. Until now, there have been no reports on effective laparoscopic approaches to the lesions suspected of GBC as well as definite GBC. Our laparoscopic approach to suspected T1 and T2 GBC is feasible and valid. It is necessary to accumulate the experience of the laparoscopic surgery for GBC and to compare the long-term results with open surgery.

Our laparoscopic approach to suspected T1 and T2 GBC is a safe and useful procedure that overcomes the risk of recurrence caused by conventional LC.

COMMENTS

Background

Laparoscopic surgery for gallbladder carcinoma (GBC) has not been widely employed yet. GBC is highly malignant, and laparoscopic surgery for GBC may increase the risk of port-site recurrence and peritoneal dissemination caused by intraoperative gallbladder injury. However, achieving the correct preoperative diagnosis and stage of GBC is very difficult. Many benign lesions including polyps and wall thickening lesions such as chronic cholecystitis and adenomyomatosis are difficult to differentiate from GBC. Such lesions are sometimes diagnosed as GBC postoperatively by pathological examination. Therefore, the above-mentioned lesions, which are associated with suspected GBC, should be carefully managed because tumor exposure as well as intraoperative gallbladder perforation can increase the risk of cancer relapse. We use a laparoscopic approach depending on the type of gallbladder lesion, including suspected T1 and T2 GBC, in this institution. In this study, the authors evaluated the usefulness of our laparoscopic approach.

Research frontiers

Laparoscopic cholecystectomy is a common procedure for treatment of benign disease. However, laparoscopic surgery for patients with suspected GBC is not recommended now due to the risk of port-site recurrence and peritoneal dissemination caused by intraoperative gallbladder perforation. This study suggested that the laparoscopic approach is feasible and useful for the management of suspected T1 and T2 GBC.

Innovations and breakthroughs

There have been no reports on effective laparoscopic approaches to the lesions suspected of GBC. The laparoscopic approach could overcome the risk of gallbladder injury which was reported to increase by laparoscopic surgery, and good results were obtained. This new laparoscopic approach to suspected T1 and T2 GBC is a safe and useful procedure.

Applications

This study suggests that our laparoscopic approach with an appropriate algorithm is useful for suspected T1 and T2 GBC. Laparoscopic surgery can be employed even in well-selected patients with T2 GBC.

Terminology

Laparoscopic whole-layer cholecystectomy (LCWL): In conventional laparoscopic cholecystectomy, the gallbladder is dissected along the inner layer of the subserosal layer, whereas in LCWL, the gallbladder is removed by dissecting along the outer layer of the subserosal layer. Laparoscopic gallbladder bed resection (LCGB): LCGB is a procedure to resect the gallbladder including 1 to 2 cm of adherent liver parenchyma laparoscopically.

Peer-review

This is a retrospective but interesting study aiming to evaluate laparoscopic surgery for "suspected" T1 and T2 gallbladder cancer. Wide spread of the laparoscopic approach has been hampered by the risk of tumor dissemination as well as by the difficulties in preoperative (and operative) diagnosis for malignancy and staging, as described by the authors. Their operative outcomes shown in the manuscript, with a precise algorithm for surgical management, are likely to be acceptable.

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ORIGINAL ARTICLE

Retrospective Study

Clinical characteristics of peptic ulcer perforation in Korea

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Author contributions: Bang CS designed research; Yang YJ, Bang CS, Shin SP, Park TY, Suk KT, Baik GH and Kim DJ performed research; Baik GH contributed new reagent/analytic tools; Yang YJ analyzed data; Yang YJ and Bang CS wrote the paper.

Institutional review board statement: This study was reviewed and approved by the institutional review board of Chuncheon Sacred Heart Hospital (2016-86).

Informed consent statement: Informed consent was exempted due to retrospective format of this study from institutional review board of Chuncheon Sacred Heart Hospital and patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

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Abstract

AIM

To elucidate the epidemiological characteristics and associated risk factors of perforated peptic ulcer (PPU).

METHODS

We retrospectively reviewed medical records of patients who were diagnosed with benign PPU from 2010 through 2015 at 6 Hallym university-affiliated hospitals.

RESULTS

A total of 396 patients were identified with post-operative complication rate of 9.1% and mortality rate of 0.8%. Among 174 (43.9%) patients who were examined for *Helicobacter pylori* (*H. pylori*) infection, 78 (44.8%) patients were positive for *H. pylori* infection, 21 (12.1%) were on non-steroidal anti-inflammatory drugs (NSAIDs) therapy, and 80 (46%) patients were neither infected of *H. pylori* nor treated by any kinds of NSAIDs. Multivariate analysis indicated that older age (OR = 1.09, 95%CI: 1.04-1.16) and comorbidity (OR = 4.11, 95%CI: 1.03-16.48) were risk factors for NSAID-associated PPU compared with non-*H. pylori*, non-NSAID associated PPU and older age (OR = 1.04, 95%CI: 1.02-1.07) and alcohol consumption (OR = 2.08, 95%CI: 1.05-4.13) were risk factors for non-*H. pylori*, non-NSAID associated PPU compared with solely *H. pylori* positive PPU.

CONCLUSION
Elderly patients with comorbidities are associated with NSAIDs-associated PPU. Non-*H. pylori*, non-NSAID peptic ulcer is important etiology of PPU and alcohol consumption is associated risk factor.

Key words: *Helicobacter pylori*; Non-steroidal anti-inflammatory drugs; Peptic ulcer perforation; Stomach ulcer; Duodenal ulcer

Core tip: The incidence of complications of peptic ulcer has not been decreasing and only a few data is available about epidemiological characteristics and associated risk factors of perforated peptic ulcer. In a retrospective review of medical records from multicenter in Korea revealed that elderly patients with comorbidities were associated with non-steroidal anti-inflammatory drugs (NSAIDs)-associated peptic ulcer perforation and non-*Helicobacter pylori* (*H. pylori*), non-NSAID peptic ulcer is important etiology in the development of peptic ulcer perforation. In a multivariate logistic regression analysis, alcohol consumption was suspected to be associated risk factors for the development of non-*H. pylori*, non-NSAID peptic perforation.

Yang YJ, Bang CS, Shin SP, Park TY, Suk KT, Baik GH, Kim DJ. Clinical characteristics of peptic ulcer perforation in Korea. *World J Gastroenterol* 2017; 23(14): 2566-2574 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i14/2566.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i14.2566

INTRODUCTION

The decreasing prevalence of *Helicobacter pylori* (*H. pylori*) infection and improvement of peptic ulcer treatment such as proton pump inhibitors (PPIs) or eradication therapies for *H. pylori* resulted in reduction of the incidence of uncomplicated peptic ulcer disease in recent decades[1-3]. However, several studies have shown controversial results showing constant incidence of complicated peptic ulcer disease[4-7], which may be due to multifactorial risk factors including the increased consumption of non-steroidal anti-inflammatory drugs (NSAIDs) or acetylsalicylic acid (ASA), especially in elderly patients with multiple comorbidities, smoking habits, or unknown etiologies[8,9].

Previous studies evaluated the epidemiologic characteristics and associated risk factors of perforated peptic ulcer (PPU) and demonstrated increasing incidence of PPU by age[7-15]. However, these studies used national registry database rather than those from hospitals, which have potential for underestimation of true incidence or misinterpretation of characteristics of PPU. Also, *H. pylori* infection status in patients with PPU was rarely evaluated except 1 study, which included suboptimal number of subjects at early 2000s[12]. Also, the effect of NSAIDs or ASA on PPU was inconsistent according to the studies[5,13,16]. Therefore, this study aimed to investigate the epidemiological characteristics and associated risk factors of benign PPU using multicenter clinical data.

MATERIALS AND METHODS

Study population

We retrospectively reviewed the medical records of 402 patients who were diagnosed with PPU (either gastric or duodenal ulcer) from January 2010 through December 2015 at Hallym university-affiliated hospitals, including the Chuncheon, Kangdong, Dongtan, Hangang, Kangnam and Hallym University Sacred Heart Hospital. Except 6 patients with unknown histology of PPU, remaining 396 ulcers were verified as benign ulcers by histology after surgical resection or endoscopic biopsy. This study was approved by the institutional review board of Chuncheon Sacred Heart Hospital (2016-86).

Data collection

We retrospectively collected the clinical data including age, sex, body mass index (BMI), smoking status and alcohol consumption for the last 3 mo, presence of any comorbidities, and current medications, such as NSAIDs or ASA, steroid, H_2-blockers, or PPIs. BMI was calculated as weight in kilograms divided by the square of height in meters. Positive alcohol consumption was defined as those who drink more than 20 g of alcohol amount in a week.

Chief complaints and laboratory data including white blood count (WBC), hemoglobin (Hb), serum creatinine (sCr), C-reactive protein (CRP) at admission period were obtained. Also, sites of perforation, treatment methods, the development of postoperative complication if surgical management was done, the length of hospital stay, and mortality rate were identified. The sites of perforation were divided into 3 areas in stomach (from cardiac to body area, proximal antrum, and from prepyloric to pyloric area) and 2 areas in duodenum (bulb, and 2nd portion). The size of perforated peptic ulcer was categorized on the basis of centimeter. The methods of operation were classified into 3 groups: (1) simple closure with or without omentopexy; (2) pyloroplasty with or without vagotomy; and (3) any other form of gastrectomy (total, subtotal, or antrectomy). If patients were assessed *H. pylori* infection status, diagnostic methods such as rapid urease test, ^{13}C-urea breath test, or the serological test and the infection status were identified. All patients who were examined for *H. pylori* were discontinued PPIs or H_2-blockers at least 4 wk before *H. pylori* test. Treatment regimen and whether the treatment was successful or not were also identified.

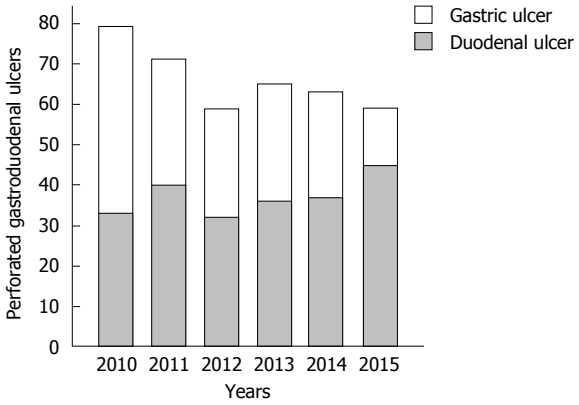

Figure 1 Annual incidence of perforated peptic ulcer according to anatomic location.

Statistical analysis

Continuous variables were expressed as mean ± SD. Categorical variables were expressed as number and percentage. We compared the differences in the clinical characteristics and therapeutic outcomes of the study population using the Student's *t*-test for the continuous variables and the Fisher's exact test for the categorical variables. To identify the risk of non-*H. pylori*, non-NSAIDs associate PPU, we performed univariate and subsequent multivariate logistic regression analysis. In this study, a P value < 0.05 (2-tailed) was adopted as the threshold of statistical significance for all tests. All of the analyses were performed using SPSS version 20.0. (SPSS Inc., Chicago, IL, United States).

RESULTS

Baseline characteristics of enrolled populations

The baseline characteristics of the study population and site specific characteristics classified according to the site of perforation are shown in Table 1. We identified a total of 396 benign PPU patients, consisting of 173 (43.7%) in gastric ulcer group and 223 (56.3%) in duodenal ulcer group. Men predominance was observed (85.1%). The mean age and BMI of the subjects were 50.6 ± 18.3 years and 21.7 ± 2.9 kg/m^2, respectively. And about half of patients had alcohol consumption (47.2%) and smoking habit (55.8%). Of all, 54 (13.6%) patients had been diagnosed with peptic ulcer at median 12 mo before the time of perforation (interquartile range: 2-36 mo). In terms of the comorbidities, 123 (31.1%) patients had at least one comorbidities, which were cardiovascular disease (67.5%), diabetes mellitus (33.3%), chronic liver disease (10.6%) and cerebrovascular disease (8.9%) in the order. The proportion of taking medication was as follows; 44 (11.2%) patients on NSAIDs including ASA (n = 23), 8 (2%) patients on steroid, and 31 (7.8%) patents on anti-ulcer medication such as PPIs (n = 19) or H_2-blocker (n = 12).

At admission, the majority of patients (92.2%) complained abdominal pain and 16 (4.0%) patients experienced melena or hematemesis. The mean levels of WBC (13.4 ± 7.8 × 10^3/uL) and CRP (93.7 ± 89.0 mg/L) were above normal range. However, the mean levels of Hb (13.7 ± 6.0 g/dL) and sCr (1.02 ± 0.7 mg/dL) were within normal value. Among 174 (43.9%) patients who were tested for *H. pylori* infection, 78 (44.8%) patients were positive for *H. pylori* tests, which were rapid urease test (n = 60), urea breath test (n = 5), and serologic test (n = 13). Comparing with the 222 patients who did not perform *H. pylori* test, patients who tested for *H. pylori* infection were significantly younger (47.6 ± 16.8 *vs* 53.0 ± 19.1 years, P = 0.003) and none of them had malignant disease. The other baseline characteristics were comparable between the patients who were tested for *H. pylori* infection or not (Table 2). Except 9 patients who were lost to follow-up, 69 (88.5%) patients were prescribed with 7 or 14 d of standard triple therapy (n = 66), or 14 d of bismuth-based quadruple therapy (n = 3) as the first-line regimen. Among them, 33 patients achieved successful eradication after the first-line treatment (eradication rate of 47.8%) and 4 patients who failed to eradication after first line regimen achieved successful eradication after 2nd line treatment (overall eradication rate of 53.6%). We could not evaluate eradication status in the remaining 32 patients due to lost to follow up during or after eradication treatment.

In terms of the site of perforation, bulb of duodenum (55.1%) was the most common site, followed by pylorus (25.3%), and antrum (15.7%). The proportion of duodenal ulcer perforation was 56.3% and the gastric ulcer perforation was 43.7%, respectively. Except 8 (2.0%) patients who were treated by medical management, remaining 388 patients (98.0%) underwent surgical management. The operative methods were primary closure with or without omentopexy (n = 307), pyloroplasty with or without vagotomy (n = 43), and any other form of gastrectomy (total, subtotal or antrectomy, n = 36). The mean duration of hospital stay was 13.1 ± 9.4 d. Though 36 (9.1%) patients experienced postoperative complication, only 3 (0.8%) patients died during hospitalization because of acute respiratory distress syndrome or uncontrolled sepsis. All of the baseline characteristics and clinical manifestations were comparable between perforated gastric ulcer and duodenal ulcer group. The detailed characteristics of all of the enrolled population are described in Tables 1 and 2.

The annual incidences of PPU showed decreasing trend for study periods, especially in gastric ulcer (Figure 1). The incidence of gastric ulcer perforation was 49.8% in the first 3 years and 36.9% in the last 3 years, which was statistically significant (P = 0.01). The decreasing incidence of perforated gastric ulcer was mainly observed in male under the age of 60. In these patients, the proportions of *H. pylori* infection, NSAIDs use, alcohol consumption, and any comorbidities were increased during study

Table 1 Baseline characteristics of enrolled population *n* (%)

Variables	Total (*n* = 396)	Gastric ulcer (*n* = 173)	Duodenal ulcer (*n* = 223)	*P* value
Sex (men)	337 (85.1)	145 (83.8)	192 (86.1)	0.57
Age	50.6 ± 18.3	51.4 ± 19.0	50.1 ± 17.8	0.49
BMI (kg/m^2)	21.7 ± 2.9	21.5 ± 2.9	21.9 ± 3.0	0.14
Alcohol consumption	187 (47.2)	76 (43.9)	111 (49.8)	0.27
Current smoking	221 (55.8)	98 (56.6)	123 (55.2)	0.84
Previous ulcer history	54 (13.6)	27 (15.6)	27 (12.1)	0.38
Comorbidity	123 (31.1)	61 (35.3)	62 (27.8)	0.13
Cardiovascular disease	83 (67.5)	38 (62.3)	45 (72.6)	0.22
DM	41 (33.3)	18 (29.5)	23 (37.1)	0.45
Chronic liver disease	13 (10.6)	8 (13.1)	5 (8.1)	0.40
Cerebrovascular disease	11 (8.9)	4 (6.6)	7 (11.3)	0.36
Malignancy	9 (7.3)	6 (9.8)	3 (4.8)	0.24
Chronic kidney injury	6 (4.9)	4 (6.6)	2 (3.2)	0.33
Pulmonary disease	4 (3.3)	3 (4.9)	1 (1.6)	0.30
Infectious disease	3 (2.4)	2 (3.3)	1 (1.6)	0.49
Current medication				
NSAIDs	44 (11.2)	20 (11.7)	24 (10.8)	0.87
Steroid	8 (2.0)	5 (2.9)	3 (1.3)	0.23
Proton pump inhibitor	19 (4.8)	11 (6.4)	8 (3.6)	0.24
H_2-blocker	12 (3.0)	5 (2.9)	7 (3.1)	> 0.99
Presentation				0.39
Abdominal pain	365 (92.2)	155 (89.6)	210 (94.2)	
Melena/hematemesis	16 (4.0)	9 (5.2)	7 (3.1)	
Shock	5 (1.3)	2 (1.2)	3 (1.3)	
Epigastric soreness	6 (1.5)	4 (2.3)	2 (0.9)	
Nausea/vomiting	4 (1.0)	3 (1.7)	1 (0.4)	
Laboratory findings				
White blood count (x10^3/uL)	13.4 ± 7.8	12.9 ± 5.3	13.8 ± 9.4	0.24
Hemoglobin (g/dL)	13.7 ± 6.0	13.7 ± 8.8	13.7 ± 2.2	0.95
Serum Creatinine (mg/dL)	1.02 ± 0.7	1.01 ± 0.7	1.03 ± 0.6	0.87
C-reactive protein (mg/L)	93.7 ± 89.0	88.9 ± 86.0	97.2 ± 91.1	0.39
Anatomical findings				
Location				
Stomach	173 (43.7)			
Body		11 (6.4)		
Antrum		62 (35.8)		
Pylorus		100 (57.8)		
Duodenum	223 (56.3)			
Bulb			218 (97.8)	
2nd portion			5 (2.2)	
Size				0.82
⩾ 1 cm	126 (37.6)	58 (38.4)	68 (37.0)	
< 1 cm	209 (62.4)	93 (61.6)	116 (63.0)	
H. pylori test				
Positivity *H. pylori* test	78/174 (44.8)	30/72 (41.7)	48/102 (47.1)	0.48
Rapid urease test	60 (76.9)	26 (86.7)	34 (70.8)	
Urea breath test	5 (6.4)	2 (6.7)	3 (6.2)	
Serology test	13 (16.7)	2 (6.7)	11 (22.9)	
Operation	388 (98.0)	170 (98.3)	218 (97.8)	0.51
Primary closure and/or omentopexy	307 (79.5)	139 (81.7)	168 (77.8)	
Pyloroplasty and/or vagotomy	43 (11.1)	15 (8.8)	28 (13.0)	
Total/subtotal gastrectomy or antrectomy	36 (9.3)	16 (9.5)	20 (9.2)	
Others (Whipple's operation, drainage)	2 (0.5)	0 (0.0)	2 (0.9)	
Medical treatment	8 (2.0)	3 (1.7)	5 (2.2)	0.09
Clinical course				
Hospital stay	13.1 ± 9.4	13.4 ± 10.4	12.8 ± 8.7	0.54
Complication	36 (9.1)	21 (12.1)	15 (6.7)	0.08
In hospital mortality	3 (0.8)	2 (1.2)	1 (0.4)	0.58

PPU: Perforated peptic ulcer; BMI: Body mass index; DM: Diabetes mellitus; NSAIDs: Non-steroidal anti-inflammatory drugs; *H. pylori*: *Helicobacter pylori*.

Table 2 Comparison of clinical characteristics between patients with perforated peptic ulcer who were tested for *Helicobacter pylori* infection or not *n* (%)

Variables	Total (*n* = 396)			Gastric ulcer (*n* = 173)			Duodenal ulcer (*n* = 223)		
	Patients who were tested for *H. pylori* infection (*n* = 174)	Patients who were not tested for *H. pylori* infection (*n* = 222)	*P* value	Patients who were tested for *H. pylori* infection (*n* = 72)	Patients who were not tested for *H. pylori* infection (*n* = 101)	*P* value	Patients who were tested for *H. pylori* infection (*n* = 102)	Patients who were not tested for *H. pylori* infection (*n* = 121)	*P* value
Sex (men)	151 (86.8)	186 (83.8)	0.480	60 (83.3)	85 (84.2)	> 0.99	91 (89.2)	101 (83.5)	0.25
Age (yr)	47.6 ± 16.8	53.0 ± 19.1	0.003	47.5 ± 18.3	54.1± 19.1	0.02	47.6 ± 15.7	52.2 ± 19.1	0.05
< 60	133 (76.4)	142 (64.0)	0.008	56 (77.8)	61 (60.4)	0.02	77 (75.5)	81 (66.9)	0.18
⩾ 60	41 (23.6)	80 (36.0)		16 (22.2)	40 (39.6)		25 (24.5)	40 (33.1)	
BMI (kg/m^2)	22.0 ± 3.1	21.5 ± 2.8	0.150	21.5 ± 3.1	21.4 ± 2.7	0.83	22.3 ± 3.0	21.6 ± 3.00	0.10
Alcohol drinking	87 (50.0)	100 (45.0)	0.360	33 (45.8)	43 (42.6)	0.76	54 (52.9)	57 (47.1)	0.42
Current smoking	97 (55.7)	124 (55.9)	> 0.99	39 (54.2)	59 (58.4)	0.64	58 (56.9)	65 (53.7)	0.69
Both alcohol consumption and smoking	69 (39.7)	85 (38.3)	0.840	24 (33.3)	36 (35.6)	0.87	45 (44.1)	49 (40.5)	0.59
Previous ulcer history	30 (17.2)	24 (10.8)	0.08	14 (19.4)	13 (12.9)	0.29	16 (15.7)	11 (9.1)	0.15
Comorbidity	44 (25.3)	79 (35.6)	0.03	21 (29.2)	40 (39.6)	0.2	23 (22.5)	39 (32.2)	0.13
HTN	27 (61.4)	55 (69.6)	0.43	14 (66.7)	24 (60.0)	0.78	13 (56.5)	31 (79.5)	0.08
DM	13 (29.5)	28 (35.4)	0.55	5 (23.8)	13 (32.5)	0.56	8 (34.8)	15 (38.5)	> 0.99
Cardiovascular disease	6 (13.6)	15 (19.0)	0.62	3 (14.3)	7 (17.5)	0.53	3 (13.0)	8 (20.5)	0.35
Chronic liver disease	5 (11.4)	8 (10.1)	0.53	1 (4.8)	7 (17.5)	0.24	4 (17.4)	1 (2.6)	0.06
Malignancy	0 (0.0)	9 (11.4)	0.02	0 (0.0)	6 (15.0)	0.07	0 (0.0)	3 (7.7)	0.24
Chronic kidney injury	3 (6.8)	3 (3.8)	0.17	2 (9.5)	2 (5.0)	0.43	1 (4.3)	1 (2.6)	0.61
Pulmonary disease	0 (0.0)	4 (5.1)	0.37	0 (0.0)	3 (7.5)	0.28	0 (0.0)	1 (2.6)	0.63
Infectious disease	1 (2.3)	2 (2.5)	0.71	1 (4.8)	1 (2.5)	0.57	0 (0.0)	1 (2.6)	0.63
Current medication									
NSAIDs	21 (12.1)	23 (10.4)	0.63	9 (12.7)	11 (11.0)	0.81	12 (11.8)	12 (9.9)	0.67
Steroid	2 (1.2)	6 (2.7)	0.24	1 (1.4)	4 (4.0)	0.31	1 (1.0)	2 (1.7)	0.56
Proton pump inhibitor	7 (4.0)	12 (5.4)	0.64	3 (4.2)	8 (7.9)	0.26	4 (3.9)	4 (3.3)	0.54
H_2-blocker	4 (2.3)	8 (3.6)	0.56	3 (4.2)	2 (2.0)	0.34	1 (1.0)	6 (5.0)	0.09

PPU: Perforated peptic ulcer; BMI: Body mass index; HTN: Hypertension; DM: Diabetes mellitus; NSAIDs: Non-steroidal anti-inflammatory drugs; *H. pylori*: *Helicobacter pylori*.

period, whereas the proportion of smoking habit was decreased from 65.9% in the first 3 years to 57.9% in the last 3 years, although the statistical significance was not reached.

Comparison of clinical characteristics and manifestations according to age

Among 396 patients, 121 (30.6%) patients were older than 60 years and the proportion of women was significantly higher in patients older than 60 years (old age group) compared with patients younger than 60 years (young age group) (5.5% *vs* 36.4%, $P < 0.001$). The proportion of alcohol consumption (56.0% *vs* 27.3%) and smoking habit (62.5% *vs* 40.5%) was higher in young age group than those of patients in old age group ($P < 0.001$). The proportions of patients with comorbidities (14.9% *vs* 67.8%) and taking NSAIDs (2.9% *vs* 30.0%) were significantly higher in old age group ($P < 0.001$), whereas the proportion of patients with *H. pylori* infection was significantly higher in young age group (50.4% *vs* 26.8%, $P = 0.008$). Although the site of perforation was comparable between two groups, the higher proportion of patients in old age group had PPU over 1 cm (31.1% *vs* 53.0%, $P < 0.001$). Moreover, the length of hospitalization (11.3 ± 7.7 *vs* 17.0 ± 11.7 d) and postoperative complication rate (4.0% *vs* 20.7%) were significantly higher in old age group ($P < 0.001$). All of the in-hospital mortality cases were also occurred in old age group (Table 3).

Comparison of clinical characteristics of PPU according to the etiology

A total of 174 patients who were tested for *H. pylori* infection status were categorized into 4 groups in terms of the etiology of peptic ulcer (both *H. pylori* positive and NSAIDs use, either *H. pylori* positive or NSAID use, and Non-*H. pylori*, non-NSAIDs group). The patients with solely *H. pylori* positive were 73 and the patients taking NSAIDs without *H. pylori* infection were 16. Five patients were infected *H. pylori* and also taking NSAIDs (Both *H. pylori* positive and NAIDs user group). The remaining 80 patients who were negative for *H. pylori* test and not taking any kinds of NSAIDs or ASA were categorized into Non-*H. pylori*, non-NSAIDs group. Men predominance was observed consistently in all of the 4 groups. The mean age (69.5 ± 12.2 years) and the proportion of patients with any comorbidities (75.0%) were significantly higher in NSAIDs user group ($P < 0.001$). The mean

Table 3 Comparison of clinical characteristics of perforated peptic ulcer according to age *n* (%)

Variables	< 60 yr (*n* = 275)	⩾ 60 yr (*n* = 121)	*P* value
Sex (men)	260 (94.5)	77 (63.6)	< 0.001
BMI (kg/m^2)	21.8 ± 2.7	21.5 ± 3.4	0.33
Alcohol consumption	154 (56.0)	33 (27.3)	< 0.001
Current smoking	172 (62.5)	49 (40.5)	< 0.001
Previous ulcer history	31 (11.3)	23 (19.0)	0.04
Comorbidity	41 (14.9)	82 (67.8)	< 0.001
Current medication			
NSAIDs	8 (2.9)	36 (30.0)	< 0.001
Steroid	4 (1.5)	4 (3.3)	0.26
Proton pump inhibitor	9 (3.3)	10 (8.3)	0.04
H_2-blocker	5 (1.8)	7 (5.8)	0.05
H. pylori test			
Positivity *H. pylori* test	67/133 (50.4)	11/41 (26.8)	0.008
Rapid urease test	52 (77.6)	8 (72.7)	
Urea breath test	4 (6.0)	1 (9.1)	
Serology test	11 (16.8)	2 (18.2)	
Anatomical findings			
Location			0.490
Gastric ulcer	117 (42.5)	56 (46.3)	
Duodenal ulcer	158 (57.5)	65 (53.7)	
Size			< 0.001
⩾ 1 cm	73 (31.1)	53 (53.0)	
< 1 cm	162 (68.9)	47 (47.0)	
Clinical course			
Hospital stay	11.3 ± 7.7	17.0 ± 11.7	< 0.001
Complication	11 (4.0)	25 (20.7)	< 0.001
In hospital mortality	0 (0.0)	3 (2.5)	0.009

PPU: Perforated peptic ulcer; BMI: Body mass index; NSAIDs: Non-steroidal anti-inflammatory drugs; *H. pylori*: *Helicobacter pylori*.

BMI level and the proportion of patients with alcohol consumption, current smoking, and peptic ulcer history were similar among the 4 groups. More than half of patients in each group experienced duodenal ulcer perforation, which were most commonly in bulb area. Also, the proportion of patients with perforation more than 1 cm in diameter was significantly higher in NSAIDs group (66.7%) than the other groups (P = 0.002). The lengths of hospital stay and postoperative complication rates were comparable among 4 group. There was no mortality during hospitalization in 4 groups. The detailed clinical characteristics of PPU according to the etiology are described in Table 4.

Associated risk factor of PPU according to the etiology

To identify the associated risk factors according to the etiology, we performed univariate and subsequent multivariate regression analysis. Older age [odds ratio (OR) = 1.09, 95% confidence interval (CI): 1.04-1.16] and comorbidity (OR = 4.11, 95%CI: 1.03-16.48) were associated with NSAID-associated PPU compared with non-*H. pylori*, non-NSAID associated PPU (Table 5). Older age (OR = 1.04, 95%CI: 1.02-1.07) and alcohol consumption (OR = 2.08, 95%CI: 1.05-4.13) were associated with non-*H. pylori*, non-NSAID associated PPU compared with solely *H. pylori* positive PPU (Table 6).

DISCUSSION

This study evaluated the clinical characteristics of PPU and assessed the associated risk factors of PPU in terms of the common etiology. Previous western studies which evaluated the incidence and changing pattern of PPU consistently revealed that most patients with PPU were aged over 60 years without gender difference and the incidence of PPU showed increasing trend by age[3,9,11]. On the other hand, a retrospective study from Middle Eastern showed that the mean age of the patients with PPU was 35.5 years and 98.3% of patients were men[13]. Also, Korean population based study using national Health Insurance claims database reported that most patients with PPU were younger than 60 years with men predominance, and increasing incidence of PPU with age, especially in women[8], which was in agreement with our study. Due to the inherent limitation of retrospective manner of this study, selection bias could be the reason of different epidemiologic characteristics. Also, there are different pattern of risk factors (*H. pylori* infection rate, NSAIDs consumption) according to the geographical area of each study. However, our study clearly categorized 4 patterns of PPU according to the etiology of peptic ulcer and each of these groups showed distinguishing characteristics of PPU.

H. pylori infected group was younger than the other groups. However, this was not due to the increased prevalence of *H. pylori* infection in younger patients. As a result of the decreasing pattern of *H. pylori* infection rate and increasing pattern of NSAIDs consumption due to the elderly society in the world as well as in Korea, NSAIDs user group was relatively older than the other groups. Korean epidemiologic studies also demonstrated the increasing age of peptic ulcer occurrence in the recent decades[17-19].

In NSAIDs users, the size of the PPU was larger than other groups and hospital stay was relatively longer than *H. pylori* infected group, although statistically insignificant. The reason of relatively larger size PPU in the NSAIDs users could not be elucidated in this study. However, considering the direct cytotoxicity in the gastric mucosa of NSAIDs other than inhibition of prostaglandin synthesis or inflammatory responses in the development of peptic ulcer, there could be a possibility of more serious injury from NSAID in the development of PPU[20].

NSAIDs users were relatively older and the proportion of women was higher than the other groups, just like the characteristics of peptic ulcer in Korea[17]. They had also more comorbidities than other groups. However, in contrast to the higher mortality rate and silent ulcer rate without significant symptoms reported in patients with NSAID induced peptic ulcer, there was no statistically significant mortality difference in NSAID induced PPU and initial symptomatic presentation was not different from those of the others[17,21].

Table 4 Comparison of clinical characteristics of perforated peptic ulcer categorized by the *Helicobacter pylori*-infected, non-steroidal anti-inflammatory drugs user, and non-*Helicobacter pylori*, non-non-steroidal anti-inflammatory drugs group *n* (%)

Variables	Both *H. pylori* positive and NSAIDs user group (*n* = 5)	*H. pylori* positive group (*n* = 73)	NSAIDs user group (*n* = 16)	Non-*H. pylori*, Non-NSAIDs group (*n* = 80)	*P* value
Sex (men)	4 (80.0)	67 (91.8)	9 (56.2)	71 (88.8)	0.005
Age	57.6 ± 16.0	40.3 ± 15.2	69.5 ± 12.2	49.1 ± 14.6	< 0.001
BMI (kg/m^2)	24.7 ± 4.1	21.6 ± 3.0	21.2 ± 4.3	22.2 ± 2.8	0.090
Alcohol consumption	3 (60.0)	33 (45.2)	4 (25.0)	47 (58.8)	0.060
Current smoking	3 (60.0)	36 (49.3)	6 (37.5)	52 (65.0)	0.090
Previous ulcer history	0 (0.0)	9 (12.3)	4 (25.0)	17 (21.2)	0.290
Comorbidity	2 (40.0)	11 (15.1)	12 (75.0)	19 (23.8)	< 0.001
Anatomical findings					
Location					0.710
Gastric ulcer	1 (20.0)	29 (39.7)	8 (50.0)	34 (42.5)	
Duodenal ulcer	4 (80.0)	44 (60.3)	8 (50.0)	46 (57.5)	
Size					0.002
⩾ 1 cm	3 (60.0)	15 (23.8)	8 (66.7)	33 (48.5)	
< 1 cm	2 (40.0)	48 (76.2)	4 (33.3)	35 (51.5)	
Clinical course					
Hospital stay	10.6 ± 4.2	10.3 ± 4.5	12.8 ± 4.8	13.2 ± 9.9	0.120
Complication	0 (0.0)	1 (1.4)	1 (6.2)	6 (7.5)	0.240
In hospital mortality	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	

PPU: Perforated peptic ulcer; BMI: Body mass index; NSAIDs: Non-steroidal anti-inflammatory drugs; *H. pylori*: *Helicobacter pylori*.

Table 5 Multivariate analysis for the risk factors of non-steroidal anti-inflammatory drugs-associated perforated peptic ulcer compared with non-*Helicobacter pylori*, non-non-steroidal anti-inflammatory drug associated perforated peptic ulcer

Variables	Unadjusted OR (95%CI)	*P* value	Adjusted OR (95%CI)	*P* value
Sex (men)	0.16 (0.05-0.55)	0.003		
Age	1.12 (1.06-1.17)	< 0.001	1.09 (1.04-1.16)	0.001
BMI (kg/m^2)	0.89 (0.74-1.07)	0.210		
Alcohol consumption	0.23 (0.07-0.79)	0.020		
Current smoking	0.32 (0.11-0.98)	0.050		
Previous ulcer history	1.24 (0.35-4.32)	0.740		
Comorbidity	9.63 (2.78-33.39)	< 0.001	4.11 (1.03-16.48)	0.050

All variables with $P \leqslant 0.2$ by univariate analysis were analyzed by multivariate analysis. BMI: Body mass index.

Table 6 Multivariate analysis for the risk factors of non-*Helicobacter pylori*, non-non-steroidal anti-inflammatory drugs perforated peptic ulcer compared with solely *Helicobacter pylori* positive perforated peptic ulcer

Variables	Unadjusted OR (95%CI)	*P* value	Adjusted OR (95%CI)	*P* value
Sex (men)	0.71 (0.24-2.09)	0.530		
Age	1.04 (1.02-1.07)	0.001	1.04 (1.02-1.07)	< 0.001
BMI (kg/m^2)	1.09 (0.97-1.22)	0.160		
Alcohol consumption	1.73 (0.91-3.28)	0.100	2.08 (1.05-4.13)	0.040
Current smoking	1.91 (1.00-3.66)	0.050		
Previous ulcer history	1.92 (0.80-4.63)	0.150		
Comorbidity	1.76 (0.77-4.00)	0.180		

All variables with $P \leqslant 0.2$ by univariate analysis were analyzed by multivariate analysis. BMI: Body mass index.

H. pylori infection and NSAIDs consumption are still important risk factors for the development of PPU. Several studies have investigated the risk factors for the development of PPU. Gisbert *et al*[12] who compared the prevalence of *H. pylori* infection and NSAIDs treatment between PPU and uncomplicated peptic ulcer disease identified that *H. pylori* prevalence were significantly lower in PPU and NSAIDs treatment was associated with PPU in a multivariate analysis. Another study from Swedish population[5] suggested that NSAIDs had little influence on peptic ulcer complications reflecting declining incidences of peptic ulcer complication despite rising NSAIDs prescription after PPI introduction. However, these studies used

suboptimal number of subjects with PPU or evaluated the effect of only NSAIDs on ulcer complication without consideration for *H. pylori* infection. Therefore, to the best of our knowledge, this is the first study which investigated the prevalence of not only patients with *H. pylori* infection and NSAIDs treatment but also with non-*H. pylori*, non-NSAIDs and included largest number of PPU patents using clinical data from hospitals.

Previous epidemiology study of peptic ulcer disease in Korea showed that there was substantial proportion of patients (40.6%) in non-*H. pylori*, non-NSAIDs peptic ulcer disease among peptic ulcers developed in a single tertiary center for a year[19], which was closely correlated with our study. In our study, almost half of the subjects (46%) were not associated with *H. pylori* infection and NSAIDs treatment and these patients had intermediate demographic characteristics between *H. pylori* infected group and NSAIDs treated group in terms of age and gender. Although the reported prevalence of non-*H. pylori*, non-NSAIDs is variable according to the geographical area due to the difference of *H. pylori* infection prevalence, previous Korean studies reported 16.2% to 22.2% of prevalence[22,23]. This rate was intensified in the development of PPU in our study, reflecting decreasing prevalence of *H. pylori* infection in Korea.

Older age and alcohol consumption were significant risk factors of non-*H. pylori*, non-NSAIDs associated PPU compared with solely *H. pylori* positive PPU, which suggested the possible effect of aging or alcohol consumption on the development of non-*H. pylori*, non-NSAIDs associated PPU. There has been few studies about the association between aging or alcohol consumption and complicated peptic ulcer disease. Andersen *et al*[15] reported that alcohol consumption was correlated with peptic ulcer bleeding, and Charpignon *et al*[24] and Xia *et al*[25] commonly showed that aging was significant risk factor for idiopathic peptic ulcers, which might be due to the association with increased comorbidities according to aging. Also, animal study suggested that decreased defense mechanism of aging such as decreased secretion of mucus, bicarbonate or prostaglandin could be the reason of peptic ulcer in elderly patients[26]. To confirm the effect of aging or alcohol consumption on PPU, further studies with large population are needed.

This study had several limitations that should be addressed. First, retrospective study design had inherently hidden bias from imperfect recall and undetectable variables. Because most patients performed only one diagnostic method to evaluate *H. pylori* status and took *H. pylori* test after the management of PPU such as antibiotics use and surgical treatment, there was possibility of false-negative results of *H. pylori* test. Also, surreptitious NSAIDs/ASA use might be underestimated the proportion of NSAIDs user group. Second, we could not verify the independent risk factors of perforated peptic ulcers by comparison between the patients with PPU and patients with uncomplicated peptic ulcer disease due to rare incidence of PPU compared with uncomplicated peptic ulcers. Third, although baseline characteristics between the patients who were tested or not for the *H. pylori* infection were comparable except age, half of the patients with PPU were not evaluated for *H. pylori* infection status because of lost to follow-up after discharge, which could affect as a selection bias. Fourth, because we initially identified the patients with PPU using ICD code and then review the medical records, there was a possibility of underestimation of mortality of PPU. Although the pitfalls stated above, this study included largest population of PPU not only patients with *H. pylori* infection and NSAIDs treatment but also with non-*H. pylori*, non-NSAIDs.

In conclusion, Elderly patients with comorbidities are associated with NSAIDs-associated PPU. Non-*H. pylori*, non-NSAID peptic ulcer is important etiology in the development of PPU and alcohol consumption is associated risk factor.

COMMENTS

Background

The incidence of complications of peptic ulcer has not decreased, and limited data are available regarding the epidemiological characteristics and associated risk factors of perforated peptic ulcers.

Research frontiers

In a retrospective review of medical records from multicenter in Korea revealed that elderly patients with comorbidities are associated with non-steroidal anti-inflammatory drugs (NSAIDs)-associated peptic ulcer perforation and non-*Helicobacter pylori* (*H. pylori*), non-NSAID peptic ulcer is important etiology in the development of peptic ulcer perforation.

Related publications

Thorsen *et al*, Epidemiology of perforated peptic ulcer: age- and gender-adjusted analysis of incidence and mortality. *World J gastroenterol* 2013; 19(3): 347-354.

Innovations and breakthroughs

In the analysis for the risk factors of non-*H. pylori*, non-NSAID peptic ulcer perforation, alcohol consumption is suspected to be associated risk factor.

Applications

Risky patients for the development of peptic ulcer perforation should be educated and managed separately according to the different etiology to prevent the serious complications of peptic ulcer.

Terminology

Non-*H. pylori*, non-NSAID peptic ulcer refers to etiologic terminology diagnosed by exclusion of common causes of peptic ulcer such as *H. pylori*, ulcerogenic drugs, and malignancy. Although clinical course of this disease entity is more serious compared with solely *H. pylori* or NSAID associated peptic ulcer, there has been no clinical recommendation in the management according to the etiology of peptic ulcer.

Peer-review

The authors elucidated the epidemiological characteristics and associated risk factors of perforated peptic ulcer in Korea. The present study was well

organized and well investigated.

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ORIGINAL ARTICLE

Retrospective Study

Effects of omeprazole in improving concurrent chemoradiotherapy efficacy in rectal cancer

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Abstract

AIM

To explore the effects of omeprazole on chemoradiotherapy efficacy and tumor recurrence in rectal cancer.

METHODS

The medical data of 125 rectal cancer patients who received the same neoadjuvant chemoradiotherapy (CRT) followed by surgery were retrospectively collected. Patients who received omeprazole (OME) orally at a dose of 20 mg at least once daily for six days and/or intravenously at 40 mg a day were recognized as eligible OME users (EOU). Otherwise, patients were regarded as non-eligible OME users (non-EOU).

Moreover, a preferred OME dose cut-off of 200 mg on tumor recurrence was obtained by receiver operating characteristic (ROC) curves. Patients were divided into two groups: the effective OME group (EOG, OME ⩾ 200 mg) and the non-effective OME group (non-EOG, OME < 200 mg).

RESULTS

The good response rate of CRT efficacy (50.8%) in EOU was significantly increased compared with non-EOU (30.6%) (P = 0.02). The recurrence rate in the EOG was 10.3%, which was significantly lower compared with 31.3% in non-EOG (P = 0.025). The good response rate of CRT efficacy in EOG was 55.2%, which was obviously higher compared with 36.5% in non-EOG, with a significant difference (P = 0.072). Multivariate Cox analysis demonstrated that OME (non-EOG and EOG) was an independent and significant impact factor for DFS (P = 0.048, HR = 0.30, 95%CI: 0.09-0.99).

CONCLUSION

When applied as an adjuvant drug in cancer treatment for relieving common side effects of chemotherapy, omeprazole has a synergetic effect in improving CRT efficacy and decreasing rectal cancer recurrence.

Key words: Omeprazole; Chemoradiotherapy efficacy; Recurrence; Rectal cancer

Core tip: In *in vitro* and *in vivo* studies, proton pump inhibitors (PPIs) induce apoptosis of gastric cancer cells, B-cell tumors and hepatoblastoma cells and promote autophagy in melanoma cells and pancreatic cancer cells. PPIs also sensitize chemo-resistant tumors to cytotoxic drugs and improve the efficacy of T-cell-based cancer immunotherapy. However, whether PPIs affect chemoradiotherapy (CRT) efficacy, decrease tumor recurrence and improve survival in rectal cancer patients remains unclear. In the present study, when used as adjuvant drug in cancer treatment, omeprazole has a synergetic effect in improving CRT efficacy and decreasing recurrences in rectal cancer.

Zhang JL, Liu M, Yang Q, Lin SY, Shan HB, Wang HY, Xu GL. Effects of omeprazole in improving concurrent chemoradiotherapy efficacy in rectal cancer. *World J Gastroenterol* 2017; 23(14): 2575-2584 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i14/2575.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i14.2575

INTRODUCTION

Rectal cancer is one of the worldwide leading causes of cancer related death[1]. Preoperative chemoradiotherapy (CRT) followed by radical surgery is a preferred treatment for patients with advanced rectal cancer for its reduced local recurrence and high sphincter preservation rate[2-4]. However, disease relapse is still a critical factor that affects patient survival[2]. The exploration of factors that affect CRT efficacy and tumor recurrence is important to improve cancer management.

Abnormal pH gradients in the tumor microenvironment are involved in tumorigenesis, tumor progression and drug resistance[5-11]. Vacuolar type H+-ATPases (V-ATPases) are proton pumps expressed on the membrane of endolysosomal organelles and plasma membranes[5], which could modulate the tumor acidic microenvironment[12,13]. V-ATPases are overexpressed in chemo-resistant cancer cells and are induced by cytotoxic drugs[14,15], playing a key role in cancer cells with a multidrug resistance phenotype[16]. Proton pump inhibitors (PPIs), such as omeprazole (OME) and esomeprazole, are used to relieve common side effects of chemotherapy, such as nausea and emesis. In addition to targeting the gastric acid pump, PPIs inhibit the activity of V-ATPases[17-20]. Moreover, PPIs induce apoptosis in gastric cancer cells[21], B-cell tumors[22] and hepatoblastoma cells[23] and promote autophagy in melanoma cells[24] and pancreatic cancer cells[25]. PPIs improve the efficacy of T-cell-based cancer immunotherapy[26-28]. In colorectal cancer, it is reported that PPIs re-sensitize drug-resistant cancer colon adenocarcinomas cell lines to cytotoxic drugs[26]

These study results suggest that the application of PPIs may be helpful in improving cancer treatment. However, whether PPIs could affect CRT efficacy, reduce tumor recurrence and improve survival in rectal cancer patients remain unclear.

MATERIALS AND METHODS

Patients

From May 2008 to March 2016, the medical records of consecutive rectal cancer patients who received the same neoadjuvant CRT followed by radical surgery were retrospectively collected. Neoadjuvant CRT included three-dimensional conformal radiotherapy (3D-CRT) using a total dose of 46 Gy concurrent with two cycles of oxaliplatin plus capecitabine. The disease was diagnosed by a combination of medical history, physical examination, biopsy, and staging examination, including abdominal ultrasound, abdominal-pelvis computed tomography, colonoscopy and endoscopic or trans-rectal ultrasonography. Tumors were staged according to the AJCC (2010 edition). Tumor stages before CRT and after surgery were classified as cTNM and ypTNM, respectively. Patients lacking detailed medical records or those with a second tumor or distant metastasis were excluded. Finally 125 patients met the criteria. The patients were aged 15-78 years, with a mean age of 55.8 ± 12.01 years. The mean

body weight and mean height of the patients was 60.1 ± 9.3 kg and 164.1 ± 6.85 cm, respectively. Pre-treatment serum carcinoembryonic antigen (CEA) and CA19-9 data were available in 120 of the 125 patients. The study was approved by the Medical Ethics Committee of Sun Yat-Sen University Cancer Center. Written informed consent was obtained from all patients.

Neoadjuvant concurrent CRT

Radiation treatment planning was designed according to the three-dimensional conformal radiation therapy (3D-CRT), with one posterior field and two lateral fields. Patients were treated using a range of 6-15 MV photons. Radiation was delivered at a total dose of 46 Gy (23 fractions with 2 Gy per fraction in 5 wk). Gross tumor volumes (GTVs) included rectal tumors and enlarged lymph nodes. Clinical target volumes (CTVs) included lymphatic drainage areas around the rectum and sacrum. Planning target volume (PTV) included areas with a 0.8-1.0 cm radial margin around the CTV. Patients were treated in the prone position, and a belly board was used to exclude the small bowel out of the radiation field. Oxaliplatin (130 mg/m^2) was delivered intravenously over 2 h on the first day of radiation treatment and on day 21. Capecitabine was administered orally twice daily at 1000 mg/m^2 on days 1-14 and days 21-34.

Dosage of omeprazole

Omeprazole usage was recorded in detail. Omeprazole was administered orally at 20 mg twice a day (Omeprazole Magnesium Entericcoated Tablets, AstraZeneca AB), 40 mg (Omeprazole Sodium for Injection, AstraZeneca AB) or 60 mg (Omeprazole Sodium for Injection, Changzhou Siyao Pharmaceuticals Co., Ltd.) intravenously one hour before the start of chemotherapy and was continuously administered in the following days if the patients complained of digestive discomfort. The reduction in gastric peak acid secretion after continuous oral administration of 20 mg OME once daily for six days was comparable with the effect of a single intravenous dose of 40 mg OME[29]. Thus, patients who received 20 mg OME orally at least once a day for six days and/or intravenous infusion of 40 mg OME daily were recognized as eligible OME users (EOU); otherwise, the patients were regarded as non-eligible OME users (non-EOU). Among the 125 patients, 63 patients met the criteria as EOU. Moreover, the bioavailability of oral enteric-coated omeprazole granules was initially low (approximately 35%-40%); however, it increased to approximately 65% on repeated dosing[30-33]. Therefore, the oral dose of EOU was multiplied by 65% to convert to a dose comparable with the intravenous dose for the intention of equal drug bioavailability.

Surgery, tumor regression evaluation and adjuvant chemotherapy

Radical surgery was performed 4-6 wk after CRT completion. Primary tumor regression grade (TRG) was determined semiquantitatively according to a modified Dworak scale[34] based on the amount of viable tumor *vs* the amount of fibrosis as follows: 0, no regression; 1, dominant tumor mass with obvious fibrosis and/or vasculopathy; 2, dominantly fibrotic changes with few tumor cells or groups (easy to find); 3, very few (difficult to find microscopically) tumor cells in fibrotic tissue with or without a mucous-like substance; and 4, no tumor cells and only fibrotic mass (total regression or response). A Dworak grade of 2 or 3 was determined by two experienced pathologists. CRT efficacy was classified as either a "good response" or a "poor response". Good response cases were those whose tumor regression was classified as TRG 3 or 4; poor response cases were those whose tumor regression was graded as TRG 0, 1 or 2. Patients were advised to undergo four to six cycles of adjuvant chemotherapy that was the same as neoadjuvant chemotherapy 4-6 wk after surgery completion. When patients could not endure the side effects of adjuvant chemotherapy, capecitabine monotherapy was adopted. Finally, 125 patients received 479 cycles of adjuvant chemotherapy.

Follow-up

After completion of combined treatment, patients were followed up every 3 to 6 mo in the first 3 years and every 12 mo thereafter. Patient evaluation included a physical examination, abdominal ultrasonography or computed tomography scan, chest X-ray, and serum CEA and Ca19-9 levels. Diagnosis of recurrence was based on two types of radiologic examination with or without abnormal plasma tumor markers. Histopathological verification was performed when necessary. The survival status was verified by examination of clinical attendance records and direct telecommunication with the patient or their family in March 2016. Survival was censored at the time of the last follow-up on March 1, 2016, with a median follow-up time of 66 mo (range 17-99 mo).

End points and statistical analysis

The study end points were CRT efficacy, recurrence, disease-free survival (DFS) and overall survival (OS). DFS was defined as the interval from surgery to either confirmed recurrence or death, and OS was defined as the time interval between surgery and death.

Continuous variables were expressed as the mean ± SD. Student *t* test and χ^2 tests were used to compare differences between groups. A receiver operating characteristic (ROC) curve was plotted to identify a proper cut-off value. Kaplan-Meier analysis was used to compare survival using the log-rank test.

Table 1 Mean dose and duration of omeprazole administered orally and intravenously

OME	Cases	administered dose (mg)				OME administration (No. of days)			
		Mean ± SD	95%CI	Max	Min	Mean ± SD	95%CI	Max	Min
Oral[1]	7	260.0 ± 143.2	127.6-392.4	546	182	11.0 ± 8.0	3.6-18.3	28	7
IV[2]	47	217.2 ± 184.8	162.8-271.3	940	40	3.8 ± 3.0	2.9-4.6	16	1
IV + Oral[3]	9	406.2 ± 184.9	264.1-548.4	756	151	13.7 ± 7.0	8.2-19.1	28	7

[1]Oral OME multiplied by 65%; [2]OME received intravenously; [3]Oral OME multiplied by 65% plus OME received intravenously. OME: Omeprazole.

Table 2 Differences in the clinicopathological characteristics in eligible omeprazole users and non-eligible omeprazole users

Characteristics	Total	EOU		*P* value
		Non	Yes	
Sex				
Male	90	46	44	0.59
Female	35	16	19	
Age (yr)				
< 60	73	37	36	0.77
⩾ 60	52	25	27	
BMI				
< 25	100	47	53	0.25
⩾ 25	25	15	10	
Tumor size (cm)				
⩽ 3	49	24	25	0.95
3-6	61	30	31	
⩾ 6	15	8	7	
Tumor grade				
1	28	14	14	0.23
2	88	46	42	
3	9	2	7	
cTNM				
Ⅱ	39	22	17	0.31
Ⅲ	86	40	46	
CEA (ng/mL)				
< 5	62	28	34	0.47
⩾ 5	58	30	28	
CA19-9 (U/mL)				
< 35	102	50	52	0.72
⩾ 35	18	8	10	
TGR				
0	39	23	16	0.25
1	15	8	7	
2	20	12	8	
3	24	9	15	
4	27	10	17	
CRT efficacy				
Poor	74	43	31	0.02
Good	51	19	32	
ypTNM				
ypcr	25	9	16	0.34
Ⅰ	26	16	10	
Ⅱ	40	20	20	
Ⅲ	34	17	17	
Adjuvant CT				
No	21	9	12	0.5
Yes	104	53	51	
Recurrence				
No	92	46	46	0.66
Yes	33	16	17	

EOU: Eligible OME users; non-EOU: Non-eligible OME users; BMI: Body mass index; TGR: Tumor regression grade; adjuvant CT: Adjuvant chemotherapy.

Univariate and multivariate Cox proportional hazard models were used to assess the effect of risk factors on survival. Forward conditional methods were used to establish the multivariate Cox proportional hazards model. A two-tailed *P* value less than 0.05 was considered statistically significant. Statistical analysis was performed using the SPSS statistical software package (version 22).

RESULTS

Clinicopathological characteristics of patients treated at different doses of OME

Among 63 OME users, 7 patients only received OME orally, 47 patients only received OME intravenously, and 9 patients received OME both orally and intravenously. The detailed information of OME dosage is presented in Table 1. The good response rate (50.8%) in the EOU was significantly increased compared with non-EOU (30.6%) (*P* = 0.02, OR = 2.336, 95%CI: 1.124-4.856). No significant differences for other clinicopathological factors were found between the EOU and non-EOU groups (all *P* values > 0.05). The patient characteristics of EOU and non-EOU are summarized in Table 2.

PPIs inhibit cancer cell proliferation in a dose-dependent manner[25,35]. Therefore, in addition to arbitrarily applying a cut-off that meets the inclusion criterion, a preferred OME dose cut-off for tumor recurrence was investigated by ROC curves. The dose that was closest to the upper left corner (100% sensitivity and 100% specificity) was selected as the cut-off dose. The area under the ROC curve (AUC) was calculated to estimate the discriminatory power of the produced OME dose cut-off of the entire dose range on recurrence. A dose cut-off of 200 mg was identified by ROC as the optimized point that differentiated recurrence from non-recurrence with maximal sensitivity and specificity (Figure 1). The AUC was 0.66 (*P* = 0.053), and the OME dose of 200 mg differentiated recurrence from non-recurrence with a specificity of 82.4% and a sensitivity of 56.5%. Patients were then divided into the effective OME group (EOG, patients received OME ⩾ 200 mg) and non-effective OME group (non-EOG, patients received OME < 200 mg). Non-EOG and EOG patient characteristics are summarized in Table 3.

Table 3 Differences in clinicopathological characteristics of non-eligible omeprazole users and eligible omeprazole users

Characteristics	Total	EOG		*P* value
		Non	Yes	
Sex				
Male	90	71	19	0.380
Female	35	25	10	
Age(yr)				
< 60	73	58	15	0.410
⩾ 60	52	38	14	
BMI				
< 25	100	77	23	0.920
⩾ 25	25	19	4	
Tumor size (cm)				
⩽ 3	49	37	12	0.940
3-6	61	47	14	
⩾ 6	15	12	3	
Tumor grade				
1	28	22	6	0.960
2	88	67	21	
3	9	7	2	
cTNM				
Ⅱ	39	30	9	0.980
Ⅲ	86	66	20	
CEA (ng/mL)				
< 5	62	45	17	0.390
⩾ 5	58	46	12	
CA19-9 (U/mL)				
< 35	102	76	26	0.420
⩾ 35	18	15	3	
TGR				
0	39	34	5	0.330
1	15	11	4	
2	20	16	4	
3	24	17	7	
4	27	18	9	
CRT efficacy				
Poor	74	61	13	0.072
Good	51	35	16	
ypTNM				
ypcr	25	16	9	0.380
Ⅰ	26	21	5	
Ⅱ	40	31	9	
Ⅲ	34	28	6	
Adjuvant CT				
No	21	14	7	0.230
Yes	104	82	22	
Recurrence				
No	97	66	26	0.025
Yes	28	30	3	

EOG: Effective OME group; non-EOG: Non-effective OME group; BMI: Body mass index; TGR: Tumor regression grade; adjuvant CT: Adjuvant chemotherapy.

The recurrence rate in EOG was 10.3% (3/29), which was significantly lower than 31.3% (30/96) in non-EOG (P = 0.025, OR = 0.25, 95%CI: 0.07-0.90; Table 3). The response rate of CRT efficacy in EOG was 55.2% (16/29), which was obviously increased compared with 36.5% (35/96) in non-EOG, with a marginally significant difference (P = 0.072, OR = 2.15, 95%CI: 0.93-5.00; Table 3). There was no significant difference in other clinicopathological features between the non-EOG and EOG groups (all P > 0.05, Table 3). Non-EOG received a total of 371 cycles of adjuvant chemotherapy, with a mean value of 3.9 ± 2.2. EOG received 108 cycles, and the mean value was 3.7 ± 2.6. The mean adjuvant chemotherapy cycles were not significantly different (P = 0.77) between the EOG and non-EOG groups.

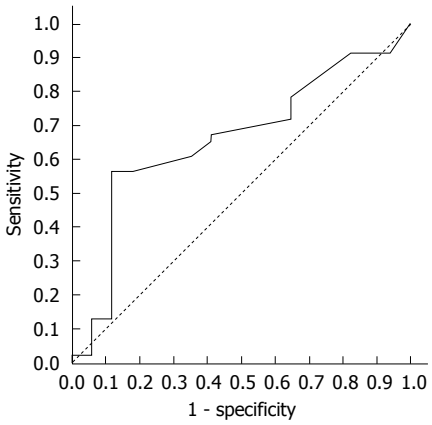

Figure 1 Receiver operating characteristic curve of omeprazole dose for recurrence.

Survival difference between the non-EOG and EOG

At the end of the study, 96 (76.8%) patients were still alive. The patients who did not survive all died from tumor-related causes, and no patient died of PPI-related severe infection[36] during the CRT treatment. The mean DFS and mean OS of all patients was 62.9 mo ± 25.5 mo, 95%CI: 58.4-67.4) and 66.6 mo ± 21.8 mo, 95%CI: 62.8-70.5), respectively. The 3- and 5-year DFS rates of all patients were 81.6% and 75.1%, respectively. The 3- and 5-year OS rates of all patients were 85.6% and 78.8%, respectively.

A significant difference in DFS was noted between non-EOG and EOG patients (P = 0.032; Figure 2A, Table 4). In addition, a marginally significant difference in OS was also observed (P = 0.092; Figure 2B and Table 4). BMI, ypTNM and CRT efficacy were significantly associated with DFS (P = 0.024, P < 0.005 and P = 0.031, respectively; Table 4), whereas cTNM was a marginally significant factor of DFS (P = 0.067; Table 4). ypTNM was the only significant impact factor of OS (P = 0.003; Table 4), and BMI was a marginally significant factor of OS (P = 0.05; Table 4).

Cox proportional hazards model analysis

The univariate Cox analysis revealed that OME (non-EOG and EOG), BMI, CRT efficacy, and ypTNM were significantly associated with DFS (P = 0.044, 0.039, 0.036 and P = 0.006, respectively; Table 5). The cTNM was significantly associated with DFS (P = 0.075; Table 5), and BMI was marginally significantly associated with OS (P = 0.069; Table 5). ypTNM was a significant impact factor for OS (P = 0.045). No other clinicopathological features significantly associated with DFS and OS (all P > 0.05; Table 5).

Furthermore, multivariate Cox analysis demon-

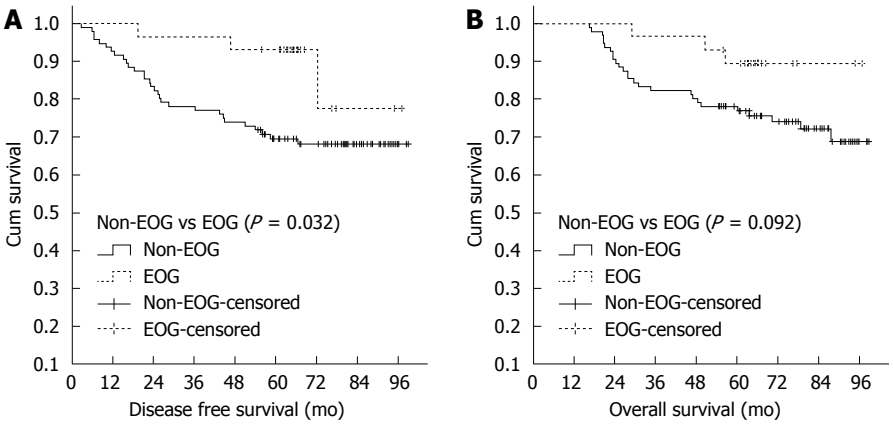

Figure 2 Disease-free survival curves (A) or OS curves (B) of non-omeprazole and omeprazole. EOG: Effective omeprazole.

Table 4 Univariate analysis of impact of various characteristics on patient survival

Characteristics	*n*	DFS			*P* value	OS			*P* value
		Mean (mo)[1]	3-yr[2]	5- yr[2]		Mean (mo)[1]	3- yr[2]	5- yr[2]	
Sex									
Male	90	61.8 ± 25.9	81.1%	74.4%	0.803	65.5 ± 22.2	84.4%	79.9%	0.855
Female	35	65.6 ± 24.6	82.9%	76.9%		69.5 ± 20.1	88.6%	79.8%	
Age (yr)									
< 60	73	63.3 ± 26.3	80.1%	71.1%	0.533	68.4 ± 22.0	86.3%	80.7%	0.908
⩾ 60	52	62.4 ± 24.6	82.7%	80.7%		64.2 ± 21.5	84.6%	78.7%	
Tumor size (cm)									
⩽ 3	48	62.5 ± 26.1	81.2%	77.0%	0.571	65.0 ± 22.4	83.3%	79.2%	0.962
> 3	77	63.2 ± 25.3	81.8%	74.0%		67.7 ± 21.6	87.0%	80.3%	
BMI									
< 25	100	60.5 ± 26.8	77.0%	69.9%	0.024	65.3 ± 22.7	82.0%	76.9%	0.050
⩾ 25	25	72.7 ± 16.6	96.0%	96.0%		72.1 ± 17.0	96.0%	92.0%	
Tumor grade									
1	28	64.7 ± 28.0	78.6%	75.0%	0.852	69.4 ± 22.5	85.7%	78.6%	0.931
2, 3	97	62.4 ± 25.0	82.5%	75.2%		65.6 ± 21.7	85.6%	80.2%	
cTNM									
Ⅱ	39	69.2 ± 23.2	87.2%	84.6%	0.067	71.9 ± 18.8	92.3%	87.2%	0.137
Ⅲ	86	60.0 ± 26.2	79.1%	70.7%		64.2 ± 22.8	82.6%	76.4%	
CEA (ng/mL)									
< 5	62	77.0 ± 4.1	69.2%	69.2%	0.789	79.6 ± 3.7	80.6%	73.9%	0.384
⩾ 5	58	80.4 ± 4.3	82.8%	74.0%		86.1 ± 3.4	89.7%	84.5%	
CA19-9 (U/mL)									
< 35	102	81.3 ± 3.1	83.3%	75.4%	0.174	84.2 ± 2.7	86.3%	80.2%	0.597
⩾ 35	18	68.1 ± 9.2	72.2%	66.7%		78.3 ± 7.8	77.8%	72.2%	
CRT efficacy									
Poor	74	60.7 ± 27.2	78.4%	67.5%	0.031	66.2 ± 23.2	83.8%	75.6%	0.144
Good	51	66.1 ± 23.0	90.2%	86.0%		67.3 ± 19.9	88.2%	86.1%	
ypTNM									
ypcr, Ⅰ, Ⅱ	91	66.1 ± 24.0	85.7%	82.3%	0.005	68.5 ± 20.1	89.0%	84.4%	0.041
Ⅲ	34	54.3 ± 28.0	70.6%	55.6%		61.6 ± 25.5	76.5%	67.6%	
Adjuvant CT									
No	21	60.2 ± 31.5	71.4%	71.4%	0.385	63.5 ± 26.5	76.2%	66.3%	0.229
Yes	104	63.5 ± 24.3	83.7%	75.8%		67.3 ± 20.8	87.5%	82.7%	
OME									
Non- EOU	62	70.0 ± 25.8	85.5%	75.6%	0.658	73.9 ± 21.9	90.3%	82.0%	0.754
EOU	63	55.9 ± 23.5	77.8%	74.6%		59.5 ± 19.5	82.5%	77.6%	
OME (200 mg)									
Non-EOG	96	62.0 ± 28.2	77.1%	69.6%	0.032	66.9 ± 24.1	82.3%	76.9%	0.092
EOG	29	65.9 ± 13.3	96.6%	46.7%		65.8 ± 12.0	96.6%	89.5%	

[1]Mean ± SD (mo); [2]Three or 5 years survival rate. EOU: Eligible OME users; Non-EOU: Non-eligible OME users; EOG: Effective OME group; Non-EOG: Non-effective OME group; BMI: Body mass index; adjuvant CT: Adjuvant chemotherapy.

Table 5 Univariate Cox analysis of the impact of various characteristics on patient survival

Characteristics	DFS		*P* value	OS		*P* value
	HR	95%CI		HR	95%CI	
Sex						
Male *vs* Female	0.91	0.42-1.65	0.800	0.93	0.41-2.09	0.860
Age (yr)						
< 60 *vs* ⩾ 60	0.80	0.40-1.62	0.530	1.05	0.50-2.19	0.910
BMI						
< 25 *vs* ⩾ 25	0.22	0.05-0.93	0.039	0.26	0.06-1.11	0.069
Tumor size (cm)						
⩽ 3 *vs* > 3	1.23	0.60-2.51	0.570	0.98	0.46-2.08	0.960
Tumor grade						
1 *vs* 2, 3	1.08	0.47-2.50	0.850	0.96	0.41-2.26	0.930
cTNM						
Ⅱ *vs* Ⅲ	2.23	0.92-5.41	0.075	1.96	0.80-4.80	0.144
CEA (ng/mL)						
< 5 *vs* ⩾ 5	0.91	0.46-1.81	0.790	0.72	0.34-1.51	0.390
CA199 (U/mL)						
< 35 *vs* ⩾ 35	1.77	0.77-4.08	0.180	1.30	0.50-3.40	0.600
CRT efficacy						
Poor *vs* good	0.43	0.19-0.95	0.036	0.55	0.24-1.24	0.150
ypTNM						
ypcr, Ⅰ, Ⅱ *vs* Ⅲ	1.61	1.14-2.27	0.006	1.46	1.01-2.11	0.045
Adjuvant CT						
Non *vs* yes	0.69	0.30-1.60	0.390	0.60	0.25-1.40	0.240
EOU						
Non *vs* yes	1.17	0.59-2.31	0.660	1.13	0.54-2.37	0.750
EOG						
Non *vs* yes	0.30	0.90-0.97	0.044	0.37	0.11-1.23	0.110

EOU: Eligible OME users; Non-EOU: Non-eligible OME users; EOG: Effective OME group; Non-EOG: Non-effective OME group; BMI: Body mass index; Adjuvant CT: Adjuvant chemotherapy.

strated that OME (non-EOG and EOG), BMI and ypTNM were independent and significant predictors of DFS (*P* = 0.048, HR = 0.30, 95%CI: 0.09-0.99, *P* = 0.038, HR = 0.22, 95%CI: 0.05-0.92 and *P* = 0.01, HR = 1.58, 95%CI: 1.12-2.22). ypTNM was also an independent and significant predictor of OS (*P* = 0.045, HR = 1.46, 95%CI: 1.01-2.11).

DISCUSSION

Neoadjuvant CRT could greatly improve the anus save rate and decrease local recurrence rate in advanced rectal cancer patients[2-4,37]. However, results addressing whether neoadjuvant CRT could improve survival are inconsistent[2,37]. The results of the present study showed that CRT efficacy is a significant clinicopathological factor associated with DFS (*P* = 0.031) and exhibits a favorable trend with OS (*P* = 0.144), indicating that CRT could decrease recurrence and potentially benefit OS. The results of the present study suggest that CRT efficacy is a significant clinicopathological factor associated with DFS, and this result is consistent with previous studies[2-4]. The present study results suggest that CRT has a potential benefit in OS, but is not a significant predictor. These results were consistent with the study by Sauer *et al*[37] but not with the study of Calogero Cammà *et al*[2]. As a potential chemotherapeutic agent[27,38-42], PPIs are safe to humans at high doses and with long-term treatment[37,38]. The mechanisms by which PPIs affect cancer include inhibiting V-ATPase activity[17,18], inducing apoptosis[21-23], promoting autophagy[24,25] and stimulating caspase-dependent cell death[35]. PPIs could sensitize chemo-resistant tumors to cytotoxic drugs[26] and could improve the efficacy of T-cell-based cancer immunotherapy[27,28], suggesting that PPIs may improve cancer treatment efficacy. In the present study, we found a good response rate (50.8%) in the EOU group that was significantly increased compared with the non-EOU group (30.6%) (*P* = 0.02), suggesting that OME could enhance the sensitivity of rectal cancer to concurrent CRT. We noticed that after the OME dose cut-off was increased, the good response rate of CRT efficacy between EOG (55.2%) and non-EOG (36.5%) patients exhibited a marginally significant difference (*P* = 0.072). This result was likely caused by an elevated cut-off that resulted in a decreased EOG sample size, which would reduce statistical power. To the best of our knowledge, this study is the first to investigate the effect of PPIs on CRT efficacy.

Abnormal extracellular acidic pH could enhance the invasive capacity and metastatic behavior of cancer cells[43-46]. V-ATPase is involved in pH-dependent degradation of the extracellular matrix and promotion of tumor invasion and metastasis[39,47], suggesting that inhibition of V-ATPase may prevent metastasis. Consistent with these studies, the present study

results showed that the recurrence rate in EOG patients was 10.3%, which was significantly lower compared with 31.3% in non-EOG patients (P = 0.025). In addition, a significant difference in DFS was noted between non-EOG and EOG patients (P = 0.032), and a marginally significant difference in OS was noted (P = 0.092). Further multivariate Cox analysis demonstrated that OME (non-EOG and EOG) is an independent and significant predictor of DFS (P = 0.048). These results suggest that when administered as an adjuvant chemoradiotherapy drug, OME may exert synergistic effects with concurrent CRT to reduce tumor recurrence.

Whether the plasma concentration of the including criteria for dosage of OME in the present study could affect cancer cell vitality should be further discussed. The oral intake of 20 mg OME could produce a maximal plasma concentration of 2.5 mg/mL after two hours in patients[48]. The minimum OME dosage for the inclusion criteria in the present study was 40 mg intravenously administered, achieving a plasma concentration of 5 mg/mL. In *in vitro* studies, OME dissolved in normal saline at a concentration of 1 mg/mL induces apoptosis in B-cell cancers[22] and re-sensitizes drug-resistant cancer cell lines (22 melanomas, 2 colon adenocarcinomas, 2 breast cancers and 2 ovarian carcinomas) to cytotoxic drugs[26]. In *in vivo* studies, 0.4 mg/kg OME co-administered with dichloroacetate and tamoxifen exhibit a synergistically anti-proliferative effect on cholangiocarcinoma[49]. In addition, 2 mg/kg OME combined with dichloroacetate exhibited an antitumor effect on HT1080 fibrosarcoma cells inoculated in mice[50]. ESOM (2.5 mg/kg) reduced tumor growth in SCID mice engrafted with human melanoma[35]. In the present study, the minimum OME dose per kilogram of body weight was approximately 0.67 mg/kg (40 mg/60 kg), and the mean dose per kilogram of body weight was 3.6 mg/kg (217.2 mg/60.0 kg), which were higher than the least functional dosage reported above[49].

BMI was significantly associated with DFS (P = 0.024) and was a marginally significant factor associated with OS (P = 0.05). Further multivariate Cox analysis demonstrated that BMI was an independent and significant predictor of DFS (P = 0.038), which was consistent with a previous study[51].

Our study has several limitations. Although consecutive patients were included, it is a retrospective study. In addition, the patient sample of the study was relatively small. However, the effects of OME on CRT efficacy, tumor recurrence and patient survival were first investigated in the present study, which would be helpful for randomized and controlled trials in the future.

In conclusion, when used as an adjuvant drug in cancer treatment, omeprazole has a synergetic effect on improving CRT efficacy and decreasing rectal cancer recurrence.

COMMENTS

Background

Abnormal pH gradients of tumor microenvironment are involved in tumorigenesis, tumor progression and drug resistance. Vacuolar type H+-ATPases (V-ATPases) are proton pumps expressed on the membrane of endolysosomal organelles and the plasma membrane, which could modulate the tumor acidic microenvironment. Proton pump inhibitors (PPIs), such as omeprazole (OME) and esomeprazole, are used to relieve common side effects of chemotherapy, such as nausea and emesis. In addition to targeting the gastric acid pump, PPIs inhibit the activity of V-ATPases. Moreover, PPIs induce apoptosis in multiple cancer cells and promotes cancer cell autophagy. PPIs also sensitize chemo-resistant tumors to cytotoxic drugs and improve the efficacy of T-cell-based cancer immunotherapy. These study results suggest that application of PPIs may be helpful to improve cancer treatment. However, whether PPIs affect CRT efficacy, reduce tumor recurrence and improve survival in rectal cancer patients remain unclear.

Research frontiers

The present study investigates whether omeprazole used as an adjuvant drug in cancer treatment could improve cancer treatment efficacy.

Innovations and breakthroughs

In contrast with previous *in vitro* and *in vivo* studies, the present study clinically revealed that when used as an adjuvant drug in cancer treatment, omeprazole has synergetic effects on improving CRT efficacy and reducing rectal cancer recurrence.

Applications

When used as an adjuvant drug in cancer treatment, omeprazole has a synergetic effect on improving CRT efficacy and reducing rectal cancer recurrence and is helpful in improving cancer treatment efficacy.

Peer-review

Zhang *et al* retrospectively reviewed a series of 125 patients with rectal cancer and demonstrated that omeprazole users had better prognosis in term of response and recurrence rates and disease-free survival.

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ORIGINAL ARTICLE

Clinical Trials Study

PIK3CA gene mutations in Northwest Chinese esophageal squamous cell carcinoma

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Abstract

AIM

To evaluate *PIK3CA* gene mutational status in Northwest Chinese esophageal squamous cell carcinoma (ESCC) patients, and examine the associations of *PIK3CA* gene mutations with clinicopathological characteristics and clinical outcome.

METHODS

A total of 210 patients with ESCC who underwent curative resection were enrolled in this study. Pyrosequencing was applied to investigate mutations in exons 9 and 20 of *PIK3CA* gene in 210 Northwest Chinese ESCCs. The associations of *PIK3CA* gene mutations with clinicopathological characteristics and clinical outcome were examined.

RESULTS

PIK3CA gene mutations in exon 9 were detected in 48 cases (22.9%) of a non-biased database of 210 curatively resected Northwest Chinese ESCCs. *PIK3CA* gene mutations were not associated with sex, tobacco

use, alcohol use, tumor location, stage, or local recurrence. When compared with wild-type *PIK3CA* gene cases, patients with *PIK3CA* gene mutations in exons 9 experienced significantly better disease-free survival and overall survival rates.

CONCLUSION

The results of this study suggest that *PIK3CA* gene mutations could act as a prognostic biomarker in Northwest Chinese ESCC patients.

Key words: *PIK3CA* gene mutations; Esophageal squamous cell carcinoma; Northwest Chinese; Prognostic significance

Core tip: *PIK3CA* gene mutations have been associated with various prognoses in patients with different cancers. However, no large-scale study has examined the prognostic impact of *PIK3CA* gene mutations in Northwest Chinese esophageal squamous cell carcinoma (ESCC). In this study, we quantified *PIK3CA* gene mutations *via* pyrosequencing technology using a non-biased database of 210 curatively resected ESCCs. It was found that *PIK3CA* gene mutations in Northwest Chinese ESCC are associated with favorable prognoses. It has been suggested that *PIK3CA* gene mutational status can have a potential role as a prognostic biomarker for ESCC.

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INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is a major histologic type of esophageal cancer that is one of the most aggressive malignant tumors worldwide, especially in East Asian countries, and accounts for most esophageal malignancies in China and Japan[1,2]. As one of the most commonly diagnosed cancers among men in China, the estimated number of new cases of esophageal cancer was 291238 in 2011, while the numbers of deaths was 218957 in the same year[3]; by 2015, these numbers had increased to 477900 and 375000, respectively[4]. Both the incidence and mortality rates were higher in rural areas than in urban areas. Despite the continuing development of cancer multimodality therapies, including surgery, radiotherapy, and chemotherapy, the prognosis of ESCC patients remains poor, even for those who undergo complete resection of their carcinomas[5].

Phosphatidylinositol 3-kinases (PI3Ks) are expressed as heterodimers of p110 catalytic subunits and p85 regulatory subunits that interact with phosphatidylinositol-3-phosphate at the membrane and catalyze the phosphorylation of protein kinase B (PKB, also known as AKT), which activates the downstream signaling pathway[6]. Activation of the PI3K/AKT signaling pathway plays an important role in the development of a variety of human carcinomas[7]. The catalytic subunits of PI3K are encoded by three genes (α, β, γ), with p110α subunit (*PIK3CA*) amplification being reported in a number of different tumor types. The mutant *PIK3CA* gene stimulates the AKT pathway and promotes cell growth and invasion in various types of human cancer[8,9] (Samuels, 2004 #620; Samuels, 2005 #638), including lung, breast, gastric, and colon[10-17].

PIK3CA gene mutations have also been detected in Japanese and Korean ESCCs[18,19]. Although independently associated with a poor prognosis in Chinese breast cancer patients[13], it was found to be associated with improved outcome in breast cancer patients in the United States[20]; this seeming contradiction requires an intensive study of this gene in future research. In addition, *PIK3CA* gene mutations and their prognostic role in Chinese ESCC patients have been rarely reported. We therefore quantified *PIK3CA* gene mutations in 210 samples of curatively resected ESCCs using pyrosequencing, and examined the prognostic significance of *PIK3CA* gene mutations in Northwest Chinese ESCC patients.

MATERIALS AND METHODS

Study subjects

A total of 210 patients with ESCC who underwent curative resection at the Second Affiliated Hospital of Xi'an Jiaotong University between 2009 and 2015 were enrolled in this study. Patients were observed at 1 to 3 mo intervals until either death or December 30, 2015. Tumor staging was carried out according to the 7th American Joint Committee Cancer Staging Manual[21]. Disease-free survival was defined as the length of time after surgical treatment of the cancer during which the patient survived with no sign of cancer recurrence. Cancer-specific survival was defined as the time between the date of operation and the date of death, which was confirmed to be attributable to ESCC. Overall survival was defined as the time between the date of the operation and the date of death. Written consent was obtained from each subject and the study procedures were approved by the ethical committees of the Second Affiliated Hospital of Xi'an Jiaotong University.

Genomic DNA extraction, polymerase chain reaction, and pyrosequencing of PIK3CA exon 9 and exon 20

Genomic DNA was extracted from 210 paraffin-embedded tissue specimens of surgically resected

Table 1 Two sets of primers of exon 9 and 20 of *PIK3CA* gene for polymerase chain reaction

Exon		Primers
Exon 9	Forward	5′CAAAGCAATTTCTACACGAGATCC 3′
	Reverse	5′GTAAAAACATGCTGAGATCAGCCACAT 3′
Exon 20	Forward	5′TGGAATGCCAGAACTACAATCTTT 3′
	Reverse	5′GGTCTTTGCCTGCTGAGAGTT 3′

esophageal cancers using the QIAamp DNA Mini kit (Qiagen, Hilgen, Germany) according to the manufacturer's instructions.

Polymerase chain reaction (PCR) amplifications targeting the *PIK3CA* gene (exon 9 and 20) were performed. Two sets of primers (Table 1) were used for the detection of any mutation points in exons 9 and 20 of the *PIK3CA* gene. PCR was carried out in a total volume of 20 μL. The mixture included 1x HotStarTaq buffer, 2.0 mmol/L Mg^{2+}, 0.2 mmol/L dNTP, 0.2 μmol/L of each primer, 1U HotStarTaq polymerase (Qiagen, Hilgen, Germany), and 1 μL template DNA. The cycling program for exon 9 was initial denaturation at 95 ℃ for 15 min, followed by 11 cycles at 94 ℃ for 20 s, 62 ℃-0.5 ℃ per cycle for 40 s, and 72 ℃ for 1 min. The cycling program for exon 20 was initial denaturation at 95 ℃ for 15 min, followed by 27 cycles at 94 ℃ for 20 s, 56 ℃ for 30 s, and 72 ℃ for 1 min. The PCR products were electrophoresed on agarose gels to confirm successful amplification of the 81 (exon 9) and 74 bp (exon 20) products.

PIK3CA pyrosequencing was carried out using the Pyro-Mark Q24 System (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Primers of *PIK3CA* gene (exon 9 and exon 20) for pyrosequencing are shown in Table 2.

Statistical analysis

For the statistical analysis, we used GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). The association between *PIK3CA* gene mutations and clinicopathological variables were performed using the χ^2-test or Fisher's exact probability test. All *P* values were two-tailed, with a *P*-value less than 0.01 being considered significant. Estimation of overall survival was calculated using the Kaplan-Meier method, with statistical differences analyzed *via* the log-rank test.

RESULTS

PIK3CA gene mutational status in ESCC

For 210 patients who had undergone curative resection of stage Ⅰ to Ⅲ ESCC, we examined *PIK3CA* gene mutations (exon 9 and exon 20) by pyrosequencing technology. In this study, *PIK3CA* gene mutations were only observed in exon 9 in 48 (22.9%) of 210 Northwest Chinese ESCC samples. The most common mutation of *PIK3CA* exon 9 was the c.1634A>C (p.E545A) mutation, which was present in 35 tumors, followed by c.1633G>A (p.E545K) in 13 tumors.

PIK3CA gene mutations and ESCC patient characteristics

We examined whether the influence of *PIK3CA* gene mutations on cancer-specific survival was modified by any of the evaluated clinical, pathologic, or epidemiologic variables of the ESCCs. As a result, we found that *PIK3CA* gene mutations were not significantly associated with any of the evaluated characteristics of ESCCs, namely sex (male *vs* female), tobacco use (yes *vs* no), alcohol use (yes *vs* no), tumor location (upper, middle *vs* lower thoracic), preoperative treatment (yes *vs* no), lymph node metastasis (yes *vs* no), or local recurrence (yes *vs* no) (all $P > 0.01$; Table 3).

PIK3CA gene mutations and patient survival

We assessed the influence of *PIK3CA* gene mutations on clinical outcome in Northwest Chinese patients with curatively resected ESCC. During the follow-up of the 210 patients, there were a total of 46 deaths confirmed to be attributable to esophageal cancer. The median follow-up time for censored patients was 36.5 mo. In the Kaplan-Meier analysis, patients with *PIK3CA* gene mutations experienced significantly longer disease-free survival (log rank $P = 0.0094$), cancer-specific survival (log rank $P = 0.0059$), and overall survival (log rank $P = 0.0066$) rates than those with the wild-type *PIK3CA* gene (Figure 1).

DISCUSSION

Numerous genetic and functional studies have clearly established a fundamental role for the PI3K signaling pathway in the development of neoplasia. As an oncogene in various human cancers, *PIK3CA* is one of the most genetically mutated genes in human cancers (including colorectal, brain, and gastric cancers)[22], having been displayed as mutated in various tumors, thereby making it a possible therapeutic marker. *PIK3CA* gene mutations and the subsequent activation of the PI3K/AKT pathway are considered to play a crucial role in cancer cell signaling pathways downstream of growth factors, cytokines, and other cellular stimuli in human neoplasm[6,23]. We therefore conducted this study to examine the prognostic impact of *PIK3CA* gene mutations among 210 Northwest Chinese patients with curatively resected ESCC.

In this study, we identified *PIK3CA* gene mutations in 48 out of 210 (22.9%) Northwest Chinese patients with curatively resected ESCC, which is a rate similar to that previously observed in ESCC (21%)[24], colorectal cancer (32%)[9], and breast cancer (25%-40%)[25,26], but slightly higher than that for gastric cancers (4.3%)[27] and brain tumors (5%)[28]. Additionally, we also found that c.1634A>C (p.E545A) was the dominant mutation type, which was consistent with a previous study in China[29]. The *PIK3CA* gene mutation

Table 2 Primers of *PIK3CA* gene for pyrosequencing

Exon		Primers
Exon 9 RS1	Nucleotide dispensation order	5′ CCATAGAAAATCTTTCTCCT 3′ 5′ ATCGACTACACTGACTGACTGACTGACTGACTG 3′
Exon 9 RS2	Nucleotide dispensation order	5′ TTCTCCTTGCTTCAGTGATTT 3′ 5′ ATACACATGTCAGTCAGACTAGCTAGCTAGCTAG 3′
Exon 9 RS3	Nucleotide dispensation order	5′ TAGAAAATCTTTCTCCTGCT 3′ 5′ ATAGCACTGACTGACTGACTACTGACTGACTGACTG 3′
Exon 20 RS	Nucleotide dispensation order	5′TGGAATGCCAGAACTACAATCTTT 3′ 5′GGTCTTTGCCTGCTGAGAGTT 3′

Table 3 *PIK3CA* mutations and clinicopathological characteristics in Northwest Chinese esophageal squamous cell carcinoma patients *n* (%)

Clinical, epidemiologic, or pathologic feature	Total, *n*	PIK3CA		*P* value
		Mutant	Wild-type	
All cases	210	48	162	
Sex				0.4756
Male	137 (65.3)	34 (70.8)	123 (75.9)	
Female	73 (34.7)	14 (29.2)	39 (24.1)	
Tobacco use				0.2684
Yes	149 (71.0)	31 (64.6)	118 (72.9)	
No	61 (29.0)	17 (35.4)	44 (28.1)	
Alcohol use				0.3778
Yes	175 (83.3)	38 (79.2)	137 (84.6)	
No	35 (16.7)	10 (20.8)	25 (15.4)	
Preoperative treatment				0.8467
Yes	28 (13.3)	6 (12.5)	22 (13.6)	
No	182 (86.7)	42 (87.5)	140 (86.4)	
Tumor location				0.9651
Upper thoracic	20 (9.5)	5 (10.4)	15 (9.2)	
Middle thoracic	109 (51.9)	25 (52.1)	84 (51.9)	
Lower thoracic	81 (38.6)	18 (37.5)	63 (38.9)	
Stage				0.1641
Ⅰ A	16 (7.6)	3 (6.3)	13 (8.0)	
Ⅰ B	20 (9.5)	5 (10.4)	15 (9.3)	
Ⅱ A	28 (13.3)	11 (22.9)	17 (10.5)	
Ⅱ B	44 (21.0)	10 (20.8)	34 (21.0)	
Ⅲ A	49 (23.3)	12 (25.0)	37 (22.8)	
Ⅲ B	23 (11.0)	1 (2.1)	22 (13.6)	
Ⅲ C	30 (14.3)	6 (12.5)	24 (14.8)	
Lymph node metastasis				0.2663
Yes	121 (57.6)	31 (64.6)	90 (55.6)	
No	89 (42.4)	17 (35.4)	72 (44.4)	
Local recurrence				0.7368
Yes	43 (20.5)	9 (18.8)	34 (21.0)	
No	167 (79.5)	39 (81.2)	128 (79.0)	
Prognosis				0.0885
Dead	88 (41.9)	15 (31.3)	73 (45.1)	
Survived	122 (58.1)	33 (68.7)	89 (54.9)	

frequency of ESCC in this study is slightly high when compared with those of previous studies; we believe this may be due to a difference in the patient cohorts, sample sizes, or methods used to assess *PIK3CA* gene mutation. When identifying *PIK3CA* gene mutations, other researchers typically use direct sequencing rather than the pyrosequencing used in the current study, which is a reliable high-throughput method that could be used as an alternative method for genotyping mutation studies[30]. There is also a non-electrophoretic nucleotide extension sequencing technology that can be used for mutation detection in tumors. Additionally, pyrosequencing has been shown to be more sensitive than regular sequencing in detecting EGFR and KRAS mutations in lung cancer patients[31,32]. *PIK3CA* gene mutational status was not identified as being associated with any clinicopathological characteristics of Northwest Chinese ESCC patients in our study, which is consistent with two other studies in Korea and China[19,33].

Identifying prognostic factors or biomarkers plays a crucial role in cancer research and clinical treatment[34-36]. Previous studies examining the relationship between *PIK3CA* gene mutations and prognosis in human cancers have yielded variable results and showed that *PIK3CA* gene mutational status is not associated with ESCC patient survival, although it does denote a better prognosis in breast cancer and ovarian cancer[37,38]. This discrepancy might be due to differences in tumor histologic type. We conducted this study to explore the prognostic impact of *PIK3CA* gene mutations among 210 Northwest Chinese patients with curatively resected ESCC. It was revealed that *PIK3CA* gene mutations were associated with a favorable prognosis among patients with curatively resected ESCC, suggesting *PIK3CA* gene mutational status may be a prognostic biomarker for Northwest Chinese ESCC patients that can be used to identify the clinical outcome of patients with curatively resected ESCC, which is consistent with its roles in Japanese ESCC patients[24]. Nonetheless, our findings regarding the correlation between *PIK3CA* mutations and favorable prognosis in esophageal cancer requires further confirmation by future independent studies using much larger non-biased cohorts of ESCCs.

In summary, this study suggests that *PIK3CA* gene mutations are associated with a favorable clinical outcome in operational resected ESCC, which supports the *PIK3CA* gene's role as a prognostic biomarker for ESCC. Our data correlates with that of previous studies suggesting that the acquisition of *PIK3CA* gene mutations may be an important molecular event in the etiology of a wide range of tumor types and highlights the potential broad applicability that the *PIK3CA* gene may have in the clinical outcome of human cancers. Future studies are needed to confirm this association and clarify the exact molecular mechanisms by which *PIK3CA* gene mutations affects human cancer behavior.

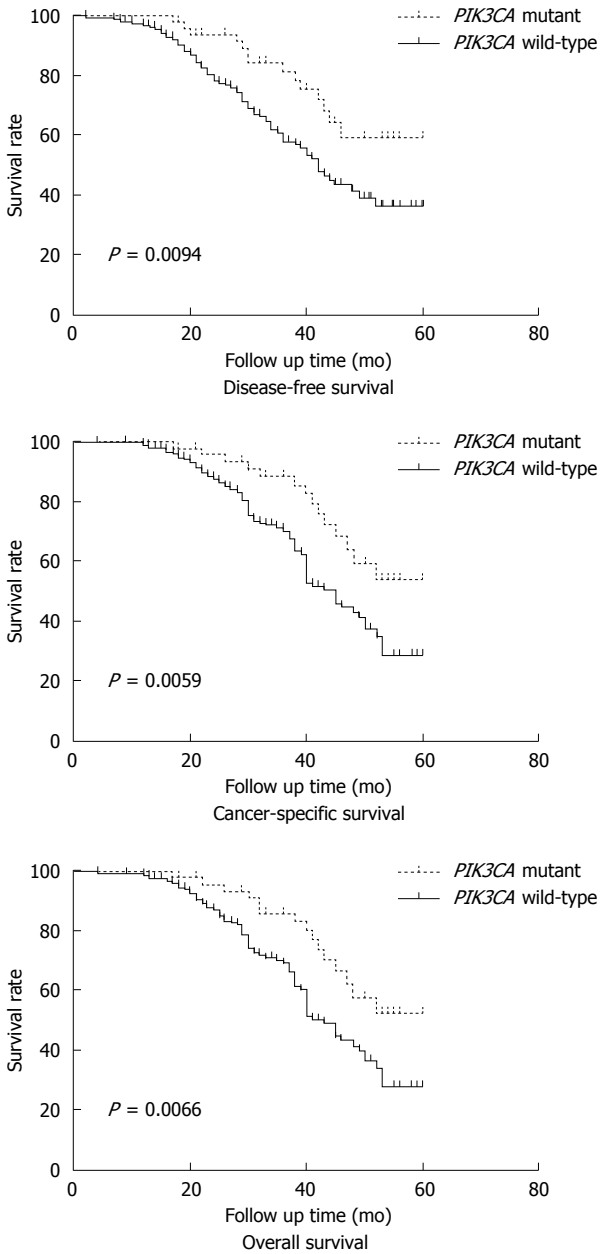

Figure 1 Kaplan-Meier curves for disease-free survival, cancer-specific survival, and overall survival in esophageal squamous cell carcinoma according to *PIK3CA* gene mutational status.

COMMENTS

Background

Esophageal squamous cell carcinoma (ESCC) is the predominant histological subtype of esophageal cancer in East Asian countries, where it accounts for more than 90% of total esophageal cancer cases. Despite the development of multimodality therapies, the prognosis of ESCC patients remains poor, even for those who undergo complete resection of their carcinomas. The 5-year survival rates of ESCC are between 11.1% and 56.5%, depending on the clinical stage at the time of diagnosis. With the development of high-throughput genome sequencing and screening technologies, an increasing number of cancer-associated genes have been identified to serve as potential therapeutic targets or prognostic indicators. High frequencies of somatic mutations conferring oncogenic potential have been found in the *PIK3CA* gene, which is associated with poor prognosis in patients with colorectal or lung cancer. In contrast, a relationship between *PIK3CA* gene mutations and favorable prognoses has been shown in breast cancer. However, no large-scale study has examined the prognostic impact of *PIK3CA* gene mutations in Northwest Chinese ESCC patients.

Research frontiers

The frequency of *PIK3CA* gene mutation in ESCC varied from 0% to 21%, which could likely introduce some bias in the statistical analyses of their clinical significance. More than 80% of *PIK3CA* gene mutations detected were localized in exons 9 and 20 (helical and kinase domain), with three "hot-spot" mutations: E542K, E545K, and H1047R. A recent report correlated with previous studies suggesting that the acquisition of *PIK3CA* mutations may be an important molecular event in the etiology of ESCC, and that mutations are associated with their clinical outcome.

Innovations and breakthroughs

This is, by far, one of the largest studies on the prognostic role of *PIK3CA* gene mutations in Northwest Chinese ESCC to date, and it shows that *PIK3CA* gene mutations in ESCC are associated with a favorable prognoses. It has been suggested that *PIK3CA* gene mutational status can have a potential role as a prognostic biomarker for ESCC patients.

Applications

PIK3CA gene mutations are associated with a favorable clinical outcome in operational resected Northwest Chinese ESCC patients, thereby suggesting that the acquisition of *PIK3CA* gene mutations may be an important molecular event in the etiology of a wide range of tumor types and highlighting the potential broad applicability that *PIK3CA* gene may have in the clinical outcome of human cancers.

Terminology

The *PIK3CA* gene is located on the 3q26.3 chromosome and encodes the catalytic p110 alpha subunit of phosphoinositide 3-kinase (PI3K). The PI3K signaling pathway is deregulated in many types of cancer, with only the *PIK3CA* gene being reported as mutated and amplified.

Peer-review

The authors examined the associations of *PIK3CA* gene mutations with clinicopathological characteristics and clinical outcome in esophageal squamous cell carcinoma patients in Northwest China. The authors exploited the most recent literature concerning the subject. The study suggests that *PIK3CA* gene mutations are associated with a favorable clinical outcome in esophageal squamous cell cancer and that in the future the evaluation of *PIK3CA* gene mutations may be potentially applied as a prognostic marker. The manuscript is worth sharing with other researchers. It is concise, clear, comprehensive, and convincing.

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ORIGINAL ARTICLE

Clinical Trials Study

Endothelial progenitor cells in peripheral blood may serve as a biological marker to predict severe acute pancreatitis

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Abstract

AIM

To investigate the significance of endothelial progenitor cells (EPCs) in predicting severe acute pancreatitis (SAP).

METHODS

We recruited 71 patients with acute pancreatitis (AP) and excluded 11 of them; finally, cases of mild acute pancreatitis (MAP) (n = 30) and SAP (n = 30), and healthy volunteers (n = 20) were internalized to investigate levels of EPCs, C-reactive protein (CRP), tumor necrosis factor-alpha (TNF-α), fibrinogen (FIB)

and white blood cells (WBC) in peripheral blood.

RESULTS

The levels of TNF-α, WBC, FIB and CRP were higher both in SAP and MAP cases than in healthy volunteers ($P < 0.05$, all). Interestingly, the level of EPCs was higher in SAP than MAP (1.63% ± 1.47% *vs* 6.61% ± 4.28%, $P < 0.01$), but there was no significant difference between the MAP cases and healthy volunteers (1.63% ± 1.47% *vs* 0.55% ± 0.54%, $P > 0.05$). Receiver operating characteristics curve (ROC) showed that EPCs, TNF-α, CRP and FIB were significantly associated with SAP, especially EPCs and CRP were optimal predictive markers of SAP. When the cut-off point for EPCs and CRP were 2.26% and 5.94 mg/dL, the sensitivities were 90.0% and 73.3%, and the specificities were 83.3% and 96.7%. Although, CRP had the highest specificity, and EPCs had the highest sensitivity and highest area under the curve value (0.93).

CONCLUSION

Data suggest that EPCs may be a new biological marker in predicting SAP.

Key words: Severe acute pancreatitis; Marker; Endothelial progenitor cells

Core tip: Endothelial progenitor cells (EPCs) may be used as a novel biological marker to predict the severity of acute pancreatitis (AP) considering the relation between endothelial cells and EPCs. We compared five markers, and concluded that EPCs had the highest area under the curve value (0.93) and Youden index (0.8), sensitivity (90.0%) and specificity (83.3%). EPCs may represent a new biological marker for predicting severe AP at the early stage.

Ha XQ, Song YJ, Zhao HB, Ta WW, Gao HW, Feng QS, Dong JZ, Deng ZY, Fan HY, Peng JH, Yang ZH, Zhao Y. Endothelial progenitor cells in peripheral blood may serve as a biological marker to predict severe acute pancreatitis. *World J Gastroenterol* 2017; 23(14): 2592-2600 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i14/2592.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i14.2592

INTRODUCTION

Acute pancreatitis (AP) is a frequent disease. Mild acute pancreatitis (MAP) is easy to treat and the cure rate is high. Although severe acute pancreatitis (SAP) accounts for only 15%-30% of AP cases, it has a high rate of multiple complications and a fatality rate of 5% to 70%[1].

The treatment strategy for SAP is different than that for MAP. The major treatment for MAP is conservative, while SAP requires enhanced monitoring and comprehensive care that includes enteral/abenteric nutrition support, antibiotics, or endoscopic sphincterotomy. Lack of accurate or timely evaluation of AP will lead to excessive medical treatments and a higher fatality rate. Therefore, correct appraisal of the severity of AP is key to clinical decision-making.

It is difficult to evaluate the severity of AP at the early stage. Over the past decade, only 19% of AP cases were graded accurately and only 67% of SAP cases received special therapy in the intensive care unit[2]. AP has a complex etiology, and disease progression does not always match clinical manifestations.

With the development of diagnostic tools - such as the Acute Physiology And Chronic Health Evaluation-Ⅱ (APACHE-Ⅱ) scoring system, Ranson criteria, the Balthazar scoring system, and the gold standard, contrast-enhanced computed tomography (CECT)[3,4] - the ability to accurately predict the severity and clinical outcome of AP patients can be up to 80%[5]. However, these methods are inconvenient to use and have limited clinical value[6-8].

The Ranson score focuses primarily on biochemical disturbances and must be completed more than 48 h after admission. The APACHE-Ⅱ score focuses on physiologic variables. APACHE-Ⅱ and Balthazar scoring can be done within the first 24 h, but the scores are based on a high number of variables, and the methods are not easily mastered. CECT also has several limitations; for example, iodinated contrast medium is contraindicated in some patients and carries the risk of nephrotoxicity. In addition, it usually requires patient transport to another hospital site.

Serum biochemical detection is objective, exact, economical, and enables real-time monitoring. Levels of tumor necrosis factor-alpha (TNF-α), white blood cell (WBC) count, C-reactive protein (CRP), and fibrinogen (FIB) all can predict SAP, yet endothelial progenitor cells (EPCs) may be a new biological marker.

Other biomarkers

Serum amylase (S-Amy) and urinary glandular amylase (U-Amy) play an important role in the final diagnosis of AP. However, the level of S-Amy in SAP may be lower than that in MAP due to extensive necrosis and calcification of the pancreas, which leads to consumption of S-Amy[9]. Therefore, it cannot be used to predict SAP exactly.

However, data show that other biomarkers, including CRP[10] and TNF-α[11], are significantly related to the severity and prognosis of AP. As a non-specific acute phase protein, CRP induces endothelial cell dysfunction, impairs vessel walls, and promotes inflammatory reactions. In addition, a certain level of CRP may impair the number and function of EPCs by depressing the expression of endothelial nitric oxide synthase mRNA[12].

In AP, TNF-α promotes a cascade of inflammatory factors, such as IL-6 and IL-1. These are produced by neutrophils and macrophages that infiltrate pancreatic tissue. IL-1 promotes aggregation of WBCs and apoptosis of pancreatic acinar cells[13].

Leukocyte-endothelial interaction and microcirculation disorder may be central to the start of AP progression[14]. In addition, both TNF-α and EPCs can promote apoptosis of pancreatic acinar cells by inducing the release of caspase-3 protease, thereby affecting the prognosis of AP[15,16]. At a certain level, TNF-α also induces premature aging of high proliferation EPCs by modulating the p38 mitogen-activated protein kinase pathway[17].

Various inflammatory mediators are produced and result in damage to cells and tissues. Highly coagulated blood also leads to microcirculation disorders and disseminated intravascular coagulation[18]. Activation of the coagulation and fibrinolytic systems cause other serious outcomes.

Damage to endothelial cells (ECs) is a key factor in systemic inflammatory mediator reactions and secondary organ injury; EPCs sustain ECs. During the embryonic period, EPCs differentiate from the outer layer of the blood-island[19]. Postnatal EPCs derive mainly from umbilical cord blood, bone marrow and peripheral blood[20].

Today, CD34+CD133+VEGFR+ cells, which are involved in neovascularization associated with angiogenic and vasculogenic mechanisms[21,22], are widely considered as EPCs[23]. As such, EPCs can also be used to predict progression or prognosis of cardiovascular diseases and tumors[24-26]. In AP, activated proteases, neutrophils and inflammatory mediums cause widespread damage to ECs, eventually leading to dysfunction of the endothelial barrier that activates coagulation and causes capillary leaks.

As stated above, damaged ECs are a critical factor in systemic inflammatory mediator reactions and secondary organ injury[27]. Previous studies report that impaired or apoptotic ECs are repaired through hyperplasia and lateral movement of peripheral mature ECs. However, in 1997, Asahara *et al*[28] first discovered that CD34+ hematopoietic stem cells were capable of differentiating into ECs and incorporating into sites of neovascularization *in vitro*.

The apoptotic bodies of ECs damaged in AP were shown to mobilize EPCs into peripheral blood from bone marrow, and to promote the proliferation and differentiation of EPCs[29]. These progressions were also shown to be mediated by inflammatory cells, *e.g.,* WBCs and macrophages, and inflammatory factors such as TNF-α, CRP and interleukin-8 (IL-8).

The purpose of this study was to investigate the significance of EPCs in predicting SAP.

MATERIALS AND METHODS

Patient recruitment

From September 2010 to October 2011, a total of 71 AP patients (38 women and 33 men; aged 22-80 years, median age of 50 years) were recruited within 24 h from the time of admission. The diagnosis of AP was made according to at least two of the following three criteria: (1) abdominal pain characteristic of AP; (2) S-Amy and/or lipase ⩾ 3 times the upper limit of normal; and (3) characteristic findings of AP on a computed tomography (CT) scan. Informed consent was obtained from the patients and ethics approval was obtained from the Institutional Research Ethics Committee.

Reports show that in tumor patients, EPCs mobilize from bone marrow into peripheral blood; besides, age and chemotherapy also affect the number of EPCs. So, exclusion criteria included any of the following: age > 80 years, a diagnosis of cancer or hematological proliferative disease under treatment, current steroid or chemotherapy for any reason, normal findings on amylase and lipase testing, failure to find changes associated with pancreatitis on CT examination, and unavailable complete blood counts or medical records. Eleven patients with AP were excluded according to these criteria.

Patients with pancreatitis were classified as the SAP group (7 women, 13 men; median age of 57 ± 16 years) if they had organ failure, a Ranson score ⩾ 3, an APACHE-Ⅱ score ⩾ 8, a class D or E Bathazar score, or a CT severity index ⩾ 4. The remainder were classified as the MAP group (11 women, 9 men; median age of 47 ± 20 years)[9].

After MAP and SAP had been diagnosed according to Chinese criteria[9], all patients received conventional treatments. Early prediction of SAP (according to EPCs, TNF-α, WBC, CRP, FIB and other criteria) was made within 24 h after admission. The control group consisted of 20 healthy volunteers (9 women and 11 men; median age of 47 years), and all of the AP patients were volunteers. In addition to the informed consent and ethics approval cited above, this study was also approved by ethics committee of Lanzhou Military Command General Hospital of the People's Liberation Army. Again, written informed consent was obtained from every subject.

Methods

We obtained blood samples from healthy volunteers and AP patients within 24 h after admission. Blood samples for cytofluorimetric analysis were processed within 12 h, whereas plasma samples were stored at -20 ℃ until used for other analyses.

Flow cytometric analysis

A total of 200 microliters of peripheral blood collected in ethylene diaminetetraacetic acid (EDTA)-containing tubes was incubated for 30 min at 4 ℃ with 5 μL of FITC-anti-CD34 and PE-anti-CD133. After red cell lysis, the samples were centrifuged and the pellets resuspended in 1 mL PBS buffer. Cells (1×10^5) were acquired by flow cytometer (FACSCalibur; Becton

Table 1 Analysis of diagnostic value

	Cut-off value	Sensitivity	Specificity	YI	AUC
EPCs, %	2.26	90.0%	83.3%	0.73	0.926
TNF-α, pg/mL	103.12	80.0%	80.0%	0.60	0.790
WBCs, 10^9/L	8.98	83.3%	63.3%	0.47	0.704
FIB, g/L	5.85	66.7%	76.7%	0.43	0.749
CRP, mg/dL	5.94	73.3%	96.7%	0.70	0.859

AUC: Area under the curve; EPCs: Endothelial progenitor cells; FIB: Fibrinogen; TNF-α: Tumor necrosis factor-alpha; WBCs: White blood cells; YI: Youden index.

Table 2 Basic characteristics of the three groups

Patient characteristic	Control group	MAP group	SAP group
Number	20	30	30
Average years of age	47.65 ± 15.14	48.17 ± 16.85	54.97 ± 15.35
Sex (male/female)	10/10	14/16	17/13

There was no significant difference in ages or sex among the three groups according to ANOVA test. MAP: Mild acute pancreatitis; SAP: Severe acute pancreatitis.

Dickinson, San Jose, CA, United States) and the percent of CD34+/CD133+ cells was analyzed using CellQuest software (BD Bioscience, San Jose, CA, United States).

Analysis of TNF-α, WBC, CRP and FIB

TNF-α was detected by enzyme-linked immunosorbent assay (ELISA) kit (R&D, Minneapolis, MN, United States). CRP was investigated by LX20 automatic biochemical analyzer (Beckman Coulter, Brea, CA, United States). WBC was analyzed by blood cell analyzer. FIB was measured by ACL 9000 automatic coagulation/fibrinolysis analyzer (Instrumentation Laboratory, Milan, Italy). To determine the diagnostic value of EPCs, TNF-α, WBC, FIB and CRP, we compared the area under the curve (AUC) and selected optimal cut-off points for distinguishing SAP from MAP. We also calculated sensitivity, specificity and the Youden index (YI) of each marker (Table 1).

Statistical analysis

We used the Statistical Package for Social Sciences (SPSS) for Windows (Version 17.0; IBM SPSS, Armonk, NY, United States). Data are shown as mean ± SD. We compared subjects using multivariate analysis of variance (ANOVA). Correlations among the five markers were analyzed using Spearman's rank correlation. We constructed receiver operating characteristic (ROC) curves taking SAP as the positive group and MAP as the negative group to predict SAP (Figure 1). The AUC was used to evaluate the diagnostic value of the five biomarkers.

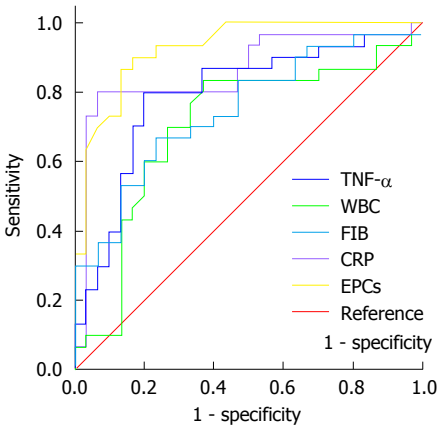


Figure 1 Receiver operating characteristic curves of endothelial progenitor cells, tumor necrosis factor-alpha, white blood cell count, fibrinogen and C-reactive protein. The severe acute pancreatitis group was taken as the positive group and the MAP group was taken as the negative group. EPCs: Endothelial progenitor cells; TNF-α: Tumor necrosis factor-alpha; WBC: White blood cell count; FIB: Fibrinogen; CRP: C-reactive protein.

RESULTS

Comparison of characteristics of SAP, MAP and control groups

The box plot shows that the distribution of data (*i.e.*, EPCs, TNF-α, WBC, FIB and CRP) in each group was asymmetrical (Figure 2). Furthermore, there were different levels of the characteristics among the three groups. Though, the level of EPCs in the control and MAP groups was similar, there was a significant difference between the MAP and SAP groups.

ANOVA showed that there was no significant difference in age or sex among the three groups. In the SAP, MAP and control groups, the serum levels of TNF-α, WBC, FIB and CRP decreased sequentially; differences were significant ($P < 0.05$, all). The level of EPCs was higher in the SAP group compared with the MAP group ($P < 0.01$), but there was no significant difference between the MAP and control groups ($P = 0.21$) (Tables 2 and 3, Figure 3).

Correlations between the five markers

Correlations between the five biomarkers were positive ($P < 0.01$, all). EPCs had the closest correlation with TNF-α ($r = 0.721$, $P = 0.00$) (Table 4, Figure 4).

Diagnostic value of EPCs, TNF-α, WBC, FIB and CRP

The optimal cut-off values of EPCs, TNF-α, FIB and CRP were 2.26%, 103.12 pg/mL, 5.85 g/L and 5.94 mg/dL, respectively. A comparison of AUCs showed AUC-EPCs (0.93) > AUC-CRP (0.86) > AUC-TNF-α (0.79) > AUC-FIB (0.75) ($P < 0.01$, all). Although AUC-WBC was 0.704 (AUC > 0.70), WBC 8.98 × 10^9 could not be used to predict SAP, perhaps due to distortions from drugs.

According to AUC or YI, EPC may be an optimal

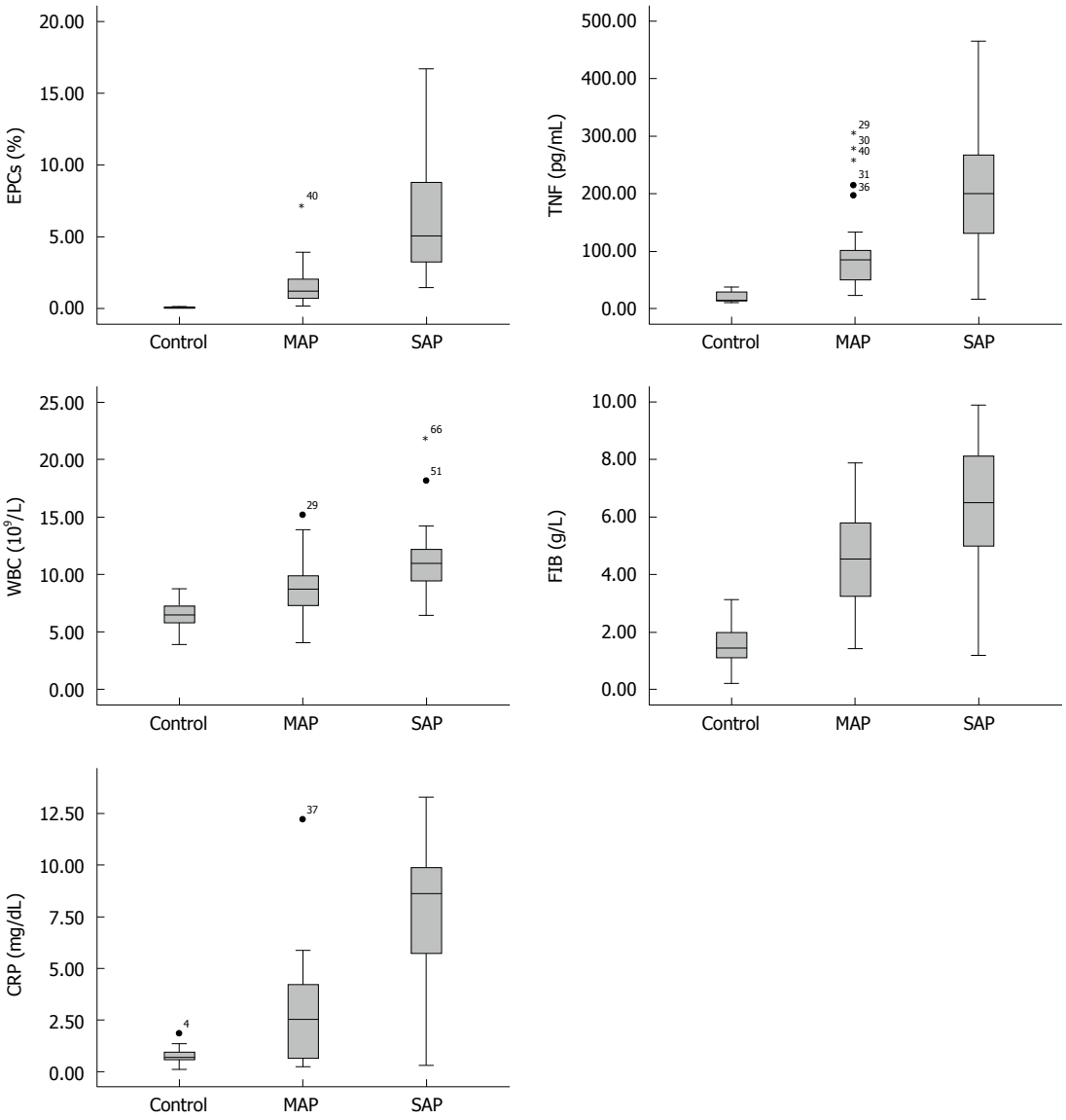


Figure 2 Distribution of data for the endothelial progenitor cells, tumor necrosis factor-alpha, white blood cell count, fibrinogen and C-reactive protein in each group was asymmetrical. EPCs: Endothelial progenitor cells; TNF-α: Tumor necrosis factor-alpha; WBC: White blood cell count; FIB: Fibrinogen; CRP: C-reactive protein.

Table 3 Comparison of the five markers in the three groups

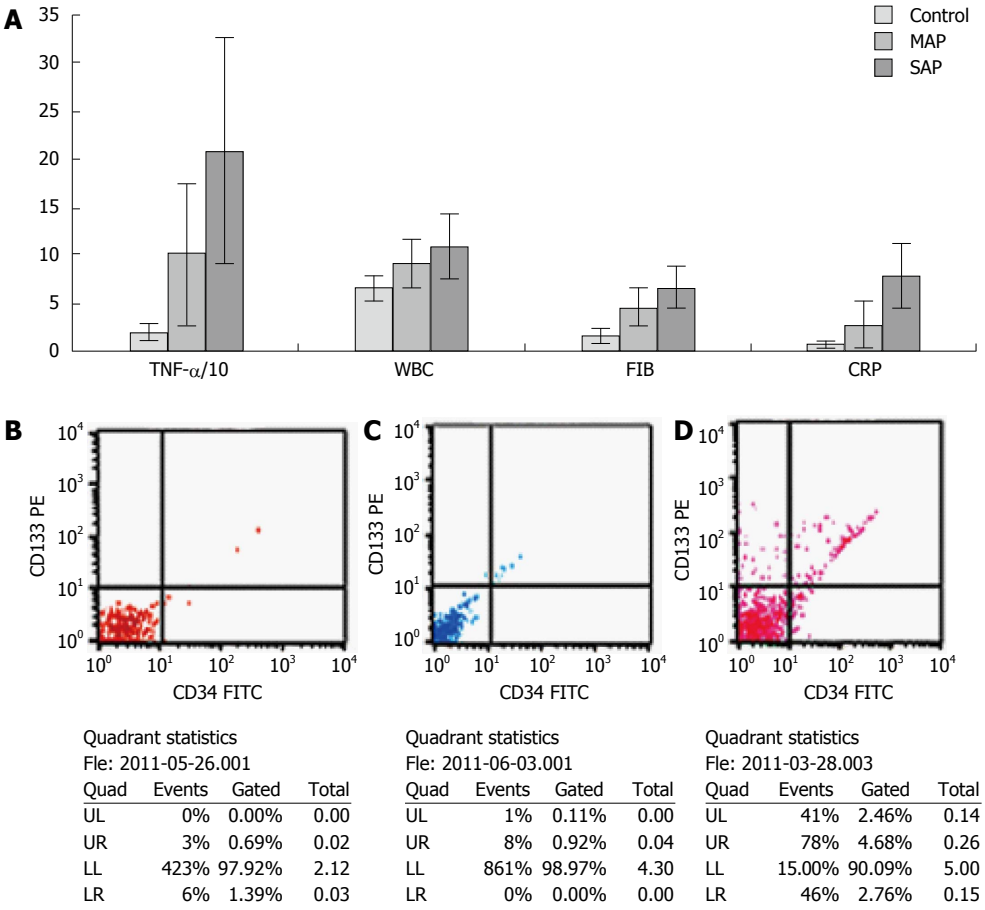
Patients characteristic	Control group	MAP group	SAP group
EPCs, %	0.55 ± 0.54	1.63 ± 1.47	6.61 ± 4.28[a,b]
TNF-α, pg/mL	19.16 ± 9.33[b]	101.18 ± 74.59[a]	208.16 ± 118.03[a,b]
WBC, 10^9/L	6.45 ± 1.24[b]	8.94 ± 2.58[a]	10.90 ± 3.47[a]
FIB, g/L	1.55 ± 0.79[b]	4.47 ± 1.85[a]	6.48 ± 2.23[a,b]
CRP, mg/dL	0.74 ± 0.40[b]	2.70 ± 2.52[a]	7.70 ± 3.36[a,b]

[a]$P < 0.05$ *vs* Control; [b]$P < 0.05$ *vs* MAP. EPCs: SAP *vs* MAP, $P = 0.00$; SAP *vs* Control, $P = 0.00$; MAP *vs* Control, $P = 0.54$; TNF-α: SAP *vs* MAP, $P = 0.00$; SAP *vs* Control, $P = 0.00$; MAP *vs* Control, $P = 0.01$; FIB: SAP *vs* MAP, $P = 0.00$; SAP *vs* Control, $P = 0.00$; MAP *vs* Control, $P = 0.00$; WBC: SAP *vs* MAP, $P = 0.07$; SAP *vs* Control, $P = 0.00$; MAP *vs* Control, $P = 0.02$; CRP: SAP *vs* MAP, $P = 0.00$; SAP *vs* Control, $P = 0.00$; MAP *vs* Control, $P = 0.04$. CRP: C-reactive protein; EPCs: Endothelial progenitor cells; FIB: Fibrinogen; TNF-α: Tumor necrosis factor-alpha; WBC: White blood cells.

marker to predict SAP, followed by CRP. Besides the highest AUC value (0.93) and YI (0.73), EPCs also had the highest sensitivity (90%), while CRP had the highest specificity (96.7%). In serial tests, the YI of combinations including EPCs was higher than that of other combinations without EPCs. EPCs combined with CRP had the highest specificity (99.4%). Combining more markers did not improve diagnostic value according to YI.

DISCUSSION

Systemic inflammatory response syndrome and multiple organ dysfunction syndrome induced by various inflammatory mediators are lethal factors in AP[30]. Inflammation and imbalance of coagulation are



Quadrant statistics
Fle: 2011-05-26.001

Quad	Events	Gated	Total
UL	0%	0.00%	0.00
UR	3%	0.69%	0.02
LL	423%	97.92%	2.12
LR	6%	1.39%	0.03

Quadrant statistics
Fle: 2011-06-03.001

Quad	Events	Gated	Total
UL	1%	0.11%	0.00
UR	8%	0.92%	0.04
LL	861%	98.97%	4.30
LR	0%	0.00%	0.00

Quadrant statistics
Fle: 2011-03-28.003

Quad	Events	Gated	Total
UL	41%	2.46%	0.14
UR	78%	4.68%	0.26
LL	15.00%	90.09%	5.00
LR	46%	2.76%	0.15

Figure 3 Contrast of the five markers. A: Comparison of the levels of tumor necrosis factor-alpha (TNF-α), white blood cell count (WBC), fibrinogen (FIB), and C-reactive protein (CRP) in the peripheral blood. The levels of TNF-α, WBC, FIB and CRP in the control, mild acute pancreatitis (MAP) and severe acute pancreatitis (SAP) groups increased in sequence; B, C, D: Flow cytometric analysis of endothelial progenitor cells (EPCs). The mean levels of EPCs in the control, MAP and SAP groups were 0.55 ± 0.54, 1.63 ± 1.47 and 6.61 ± 4.28, respectively. There was a significant difference between the MAP and SAP groups ($P < 0.01$). However, the level of EPCs in the control and MAP groups was similar.

two keys to these pathologic processes. Therefore, inflammatory and coagulation factors may serve as biological markers to predict the severity and prognosis of AP.

New biological maker to predict SAP

EPCs have a close relation with the endothelial system, and may be antigen-presenting cells[31]. That means EPCs may contribute to the processes of AP, and may be a potential marker to predict the severity and prognosis of AP at the early stage. This investigation supports that hypothesis.

Data indicate that the normal level of EPCs in peripheral blood range from 0% to 0.05% of circulating mononuclear cells[32,33]. We found that the mean level of EPCs in peripheral blood of the control, MAP and SAP patients were 0.55% ± 0.54%, 1.63% ± 1.47% and 6.61% ± 4.28%, respectively. The difference between the MAP and SAP groups was significant ($P < 0.01$). However, the level of EPCs in the control and MAP groups was similar. Furthermore, the control group level of EPCs (mean of 0.55% ± 0.54%, range of 0% to 0.16%) was higher than reported. This may be attributed to national, altitude and other factors.

Table 4 Relations among the five markers

	TNF-α, *r* value	WBCs, *r* value	FIB, *r* value	CRP, *r* value
EPCs	0.721[1]	0.594[2]	0.703[3]	0.666
TNF-α		0.555	0.639	0.614
WBCs			0.442	0.408
FIB				0.685[4]

[1]Endothelial progenitor cells (EPCs) had the closest correlation with tumor necrosis factor-alpha (TNF-α) ($P < 0.01$); [2]White blood cells (WBCs) had the closest correlation with EPCs ($P < 0.01$); [3]Fibrinogen (FIB) had the closest correlation with EPCs ($P < 0.01$); [4]C-reactive protein (CRP) had the closet correlation with FIB ($P < 0.01$).

EPCs also had positive correlations with the other four markers in AP patients and controls ($P < 0.01$, all).

According to AUC value and YI, EPCs and CRP appeared to be optimal biomarkers for predicting SAP. Although CRP had the highest specificity (96.7%), EPCs had the highest sensitivity (90.0%) and highest AUC value (0.93) compared with the other five markers. CRP is produced by the liver after the stimulation of IL-6 and other hormones so that the peak of CRP appears 24-48 h later than IL-6; as well,

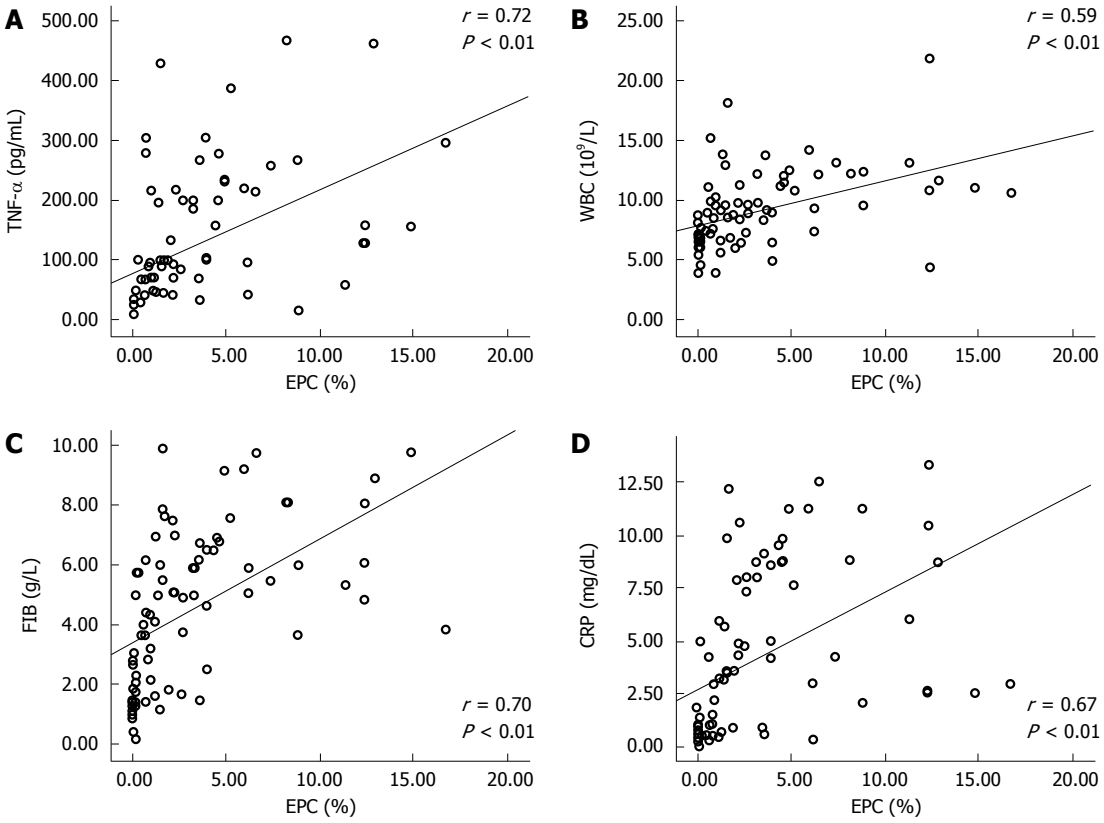


Figure 4 Spearman's correlations between endothelial progenitor cells and the other four markers showed the endothelial progenitor cells had a positive correlation with the other four markers. A-D: The closest correlation was between endothelial progenitor cells (EPCs) and tumor necrosis factor-alpha (TNF-α) (r = 0.72, P < 0.01).

the different level of CRP between MAP and SAP groups appears 2 d later, after clinical symptoms occur[34]. In contrast, EPCs are instantly mobilized, suggesting that EPCs might be superior to CRP in predicting SAP at the early stage.

CRP as a feasible biological marker to predict SAP

CRP impairs the repairing effect of EPCs and leads to the dysfunction of ECs, finally resulting in progression of AP. This investigation showed that CRP has a positive correlation with EPCs that may be attributable to the peripheral blood level of CRP. CRP is accurate, cost-effective and popular. Therefore, it could be used as a significant independent biological marker[35,36].

The World Congress of Gastroenterology also suggests that CRP might be an independent risk factor for SAP. If CRP is > 150 mg/L in 72 h, it suggests SAP and the occurrence of complications. The Congress' s Report[37] included that the sensitivity, specificity, positive predictive value (PV+) and negative predictive value (PV-) of CRP were 86%, 87%, 75% and 93%, respectively. This investigation indicates that CRP > 5.94 mg/dL (59.4 mg/L) within 24 h after admission suggests SAP. Furthermore, at this cut-off value, the sensitivity, specificity, PV+ and PV- of CRP were 73.3%, 96.7%, 95.7% and 78.4%, respectively.

CRP is produced later in the progress of AP, with the peak sustained in only 24 h. That the optimal cut-off level is lower than reported, may be attributed to different patient admission times.

Value of TNF-α and FIB is still in dispute

TNF-α rose rapidly at the early stage of AP, and it had a negative correlation with the rate of decay and the severity of AP. This investigation indicated that TNF-α, with a significant AUC value (AUC-TNF-α > 0.7), can be used as a marker to predict SAP at the early stage.

Since coagulation function disorder also occurs in the early stage of SAP, markers of coagulation function can also be used to predict the severity of AP. FIB is the most important coagulation factor, with the highest normal serum level of 2-4 g/L. Reports suggest that progressive change indicates poor prognosis. This investigation found that FIB > 5.85 g/L may predict SAP with a respective sensitivity and specificity of 66.7% and 76.7%.

According to the AUC value and YI, both TNF-α and FIB seemed to have lower diagnostic value than EPCs or CRP. Furthermore, WBC could be easily modified by anti-inflammatory drugs, such as aspirin, making it an unlikely biomarker to identify SAP or MAP.

In this investigation, we first proposed that EPCs may be used as a novel biological marker to predict the severity of AP considering the relation between ECs and EPCs. We compared five markers, and concluded that EPCs had the highest AUC value (0.93) and YI

(0.8), sensitivity (90.0%) and specificity (83.3%). According to the YI, combination of CRP with EPCs would improve diagnostic value. Data suggest that EPCs may be a new biological marker in predicting SAP at the early stage.

COMMENTS

Background

Acute pancreatitis (AP) is a frequent disease. Mild acute pancreatitis is easy to treat and the cure rate is high. Although severe acute pancreatitis (SAP) accounts for only 15%-30% of AP cases, it has a high rate of multiple complications and a fatality rate of 5% to 70%. Lack of accurate or timely evaluation of AP will lead to excessive medical treatments and a higher fatality rate. Therefore, correct appraisal of the severity of AP is key to clinical decision-making. Data show that biomarkers, including C-reactive protein (CRP) and tumor necrosis factor-alpha (TNF-α) are significantly related to the severity and prognosis of AP. As a non-specific acute phase protein, CRP induces endothelial cell dysfunction, impairs vessel walls and promotes inflammatory reactions. In addition, a certain level of CRP may impair the number and function of endothelial progenitor cells (EPCs) by depressing the expression of endothelial nitric oxide synthase. The purpose of this study was to investigate the significance of EPCs in predicting SAP.

Research frontiers

EPCs have a close relation with the endothelial system, and may be antigen-presenting cells. That means that EPCs may contribute to the processes of AP, and may be a potential marker to predict the severity and prognosis of AP at the early stage.

Innovations and breakthroughs

In this investigation, the authors first proposed that EPCs may be used as a novel biological marker to predict the severity of AP, considering the relation between ECs and EPCs. The authors compared five markers, and concluded that EPCs had the highest AUC value (0.93) and Youden index (YI) (0.8), as well as the highest sensitivity (90.0%) and the second highest specificity (83.3%) from among five markers evaluated. According to the YI, combination of CRP with EPCs will improve diagnostic value. Furthermore, this investigation showed that EPCs had positive correlations with the four other markers in AP patients.

Applications

This study suggests that EPCs may be used as a new biological marker in predicting SAP at the early stage.

Terminology

EPCs may be used as a novel biological marker to predict the severity of AP considering the relation between ECs and EPCs. The authors compared five markers, and concluded that EPCs had the highest AUC value (0.93) and YI (0.8), sensitivity (90.0%) and specificity (83.3%). EPCs may be a new biological marker in predicting SAP at the early stage.

Peer-review

This article relooks at whether EPCs may be used as a novel biological marker to predict the severity of AP, considering the relation between ECs and EPCs. Compared with five markers, the authors concluded that EPCs had the highest AUC value (0.93) and YI (0.8), sensitivity (90.0%) and specificity (83.3%). So, EPCs may be useful as a new biological marker in predicting SAP at the early stage.

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ORIGINAL ARTICLE

Clinical Trials Study

Comparative study of ROR2 and WNT5a expression in squamous/adenosquamous carcinoma and adenocarcinoma of the gallbladder

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Author contributions: Wu ZC, Xiong L and Wang LX carried out studies and wrote the paper; Yang ZL designed the study and revised the paper; Liu ZR, Liu KJ and Yang ZL performed the statistical analysis; Miao XY and Zhao H conceived of the study and participated in the design and coordination; Li DQ and Zou Q collected specimens and experimental materials; Wu ZC and Xiong L contributed equally to this work.

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Institutional review board statement: This study was approved by the Institutional Review Board of Second Xiangya Hospital, Central South University.

Clinical trial registration statement: This registration policy applies to prospective, randomized, controlled trials only.

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Abstract

AIM

To investigate the expression and clinical pathological

significance of ROR2 and WNT5a in gallbladder squamous/adenosquamous carcinoma (SC/ASC) and adenocarcinoma (AC).

METHODS

EnVision immunohistochemistry was used to stain for ROR2 and WNT5a in 46 SC/ASC patients and 80 AC patients.

RESULTS

Poorly differentiated AC among AC patients aged > 45 years were significantly more frequent compared with SC/ASC patients, while tumors with a maximal diameter > 3 cm in the SC/ASC group were significantly more frequent compared with the AC group. Positive ROR2 and WNT5a expression was significantly lower in SC/ASC or AC with a maximal mass diameter ≤ 3 cm, a TNM stage of Ⅰ + Ⅱ, no lymph node metastasis, no surrounding invasion, and radical resection than in patients with a maximal mass diameter > 3 cm, TNM stage Ⅳ, lymph node metastasis, surrounding invasion, and no resection. Positive ROR2 expression in patients with highly differentiated SC/ASC was significantly lower than in patients with poorly differentiated SC/ASC. Positive ROR2 and WNT5a expression levels in highly differentiated AC were significantly lower than in poorly differentiated AC. Kaplan-Meier survival analysis showed that differentiation degree, maximal mass diameter, TNM stage, lymph node metastasis, surrounding invasion, surgical procedure and the ROR2 and WNT5a expression levels were closely related to average survival of SC/ASC or AC. The survival of SC/ASC or AC patients with positive expression of ROR2 and WNT5a was significantly shorter than that of patients with negative expression results. Cox multivariate analysis revealed that poor differentiation, a maximal diameter of the mass ≥ 3 cm, TNM stage Ⅲ or Ⅳ, lymph node metastasis, surrounding invasion, unresected surgery and positive ROR2 or WNT5a expression in the SC/ASC or AC patients were negatively correlated with the postoperative survival rate and positively correlated with mortality, which are risk factors and independent prognostic predictors.

CONCLUSION

SC/ASC or AC patients with positive ROR2 or WNT5a expression generally have a poor prognosis.

Key words: Gallbladder adenosquamous carcinoma; Gallbladder squamous carcinoma; ROR2; Gallbladder adenocarcinoma; WNT5a; Immunohistochemistry

Core tip: Gallbladder carcinoma (GBC) is a highly aggressive malignancy of the biliary tract. However, biological markers for the diagnosis, prognosis, and targeted therapy of GBCs are still not clear. In this study, we investigated the clinicopathological significance of ROR2 and WNT5a in squamous/adenosquamous carcinoma (SC/ASC) and adenocarcinoma (AC) of the gallbladder. We found that positive ROR2 or WNT5a expression in both SC/ASC or AC patients were negatively correlated with postoperative survival rate and positively correlated with mortality. Elevated expression levels of ROR2 or WNT5a are associated with a higher risk of GBC and are independent prognostic predictors.

Wu ZC, Xiong L, Wang LX, Miao XY, Liu ZR, Li DQ, Zou Q, Liu KJ, Zhao H, Yang ZL. Comparative study of ROR2 and WNT5a expression in squamous/adenosquamous carcinoma and adenocarcinoma of the gallbladder. *World J Gastroenterol* 2017; 23(14): 2601-2612 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i14/2601.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i14.2601

INTRODUCTION

Gallbladder carcinoma is rare, accounting for approximately 0.28% of the general surgery diseases treated within a given period of time. Gallbladder carcinomas mainly consist of adenocarcinomas (ACs), which account for > 90% of gallbladder carcinomas. Gallbladder squamous carcinoma (SC) and adenosquamous carcinoma (ASC) are rare pathological subtypes of gallbladder carcinoma, with a combined incidence rate accounting for approximately 1.4%-10.4% of all gallbladder carcinomas[1]. Because of this low incidence, the clinicopathological characteristics and biological behavior of SC/ASC remain unclear.

Few studies on this topic are available in the literature, and most of them consist of case reports or clinical case analyses[1-6]. In recent years, some studies have identified the following characteristics of SC/ASC[1]. No special symptoms are shown in the early stage, with the most common clinical manifestations being right upper abdominal pain and discomfort. Most cases are in the late stage at diagnosis, and the efficacy of surgery is therefore poor. Early diagnosis and radical surgery contributes to improvement of the prognosis[2].

The disease is likely to be initiated with squamous epithelium metaplasia in the gallbladder mucosa, followed by carcinogenesis on this basis, though some researchers believe that it occurs in the pluripotent basal cells in the gallbladder mucosa; more in-depth study is awaited[3]. Carcinogenic factors may mainly be related to the long-term chronic irritation of inflammation and cholelithiasis. Some authors have also proposed that carcinogenesis may be related to the long-term effects of bacteria and chemical reactions in bile, forming carcinogens such as anthracene and methylcholanthrene[4].

A number of studies has found that compared with AC, SC/ASC shows stronger proliferation, higher malignancy and directly invades the surrounding tissues and organs much more readily, whereas it

shows less frequent regional metastasis in the lymph nodes and other distant organs compared with AC. However, this finding is still controversial, with different perspectives being discussed[5]. Surgery is still the preferred treatment for SC/ASC, but the efficacy of surgery is largely dependent on clinical staging and the applied surgical methods. In addition, postoperative radiotherapy may have some effect[6]. Few or no fundamental systematic studies on the pathogenesis of SC/ASC have been reported in the literature, and most studies are focused on markers of pathological diagnosis, due to the extremely low incidence of SC/ASC and the difficulty of collecting a given number of cases.

Receptor tyrosine kinase-like orphan receptor 2 (ROR2) belongs to the receptor tyrosine kinase (RTK) family. Many members of this kinase family play an important role in the process of morphogenesis and differentiation in mammals. ROR2 can act as a receptor for Wnt5a, leading to signal transduction through the JAK-STAT3 and Wnt/JNK signaling pathways[7,8]. Recent studies have found that the expression of ROR2 is closely related to the occurrence, progression, biological behavior and prognosis of a variety of malignant tumors, such as gastric cancer[9], colorectal cancer[10], liver cancer[11], breast cancer[12], esophageal squamous carcinoma[12] and medulloblastoma[13]. Further studies demonstrated that malignancies with a high expression level of ROR2 are typically poorly differentiated, of a high clinical stage, and prone to metastasis with strong invasiveness. ROR2 is considered to be an important biological indicator for assessing the prognosis of patients with malignant tumors, and a high expression level of ROR2 may suggest a poor prognosis.

Wnt5a is a member of the Wnt family that has received major interest in recent years. The Wnt5a protein is involved in many physiological and pathological processes, such as embryonic development, inflammation and tumor development. It plays an important role in polarity, orientation, deformation of the cell cytoskeleton and a variety of malignant processes in tumor cells. Abnormal expression of the Wnt5a protein can be observed in a variety of epithelial and mesenchymal tumors. Different experimental studies have arrived at opposite conclusions regarding whether Wnt5a is a carcinogen or a suppressor of tumor development. Wnt5a is down-regulated and plays a suppressor role in colorectal cancer[14], neuroblastoma[15] and leukemia[16]. Down-regulation of Wnt5a is positively correlated with the stage of a tumor and is an independent prognostic factor in the various tumor subtypes. In cutaneous melanoma[17], breast cancer cells[18], gastric cancer[19], non-small cell lung cancer[20] and prostate cancer[21], Wnt5a is over-expressed and affects the migration and invasiveness of the tumor, showing the characteristics of an oncogene.

No study addressing the expression levels of ROR2 and Wnt5a in gallbladder SC/ASC and AC has been reported in the literature to date. In the present study, using the EnVision immunohistochemistry method, the expression levels of ROR2 and Wnt5a and their clinical pathological significance were investigated in tissues from 46 cases of gallbladder SC/ASC and 80 cases of gallbladder AC. Additionally, the clinicopathological characteristics of SC/ASC and AC, and the differences in the expression of ROR2 and Wnt5a were compared.

MATERIALS AND METHODS

Clinical and pathological data

Surgical resection specimens from a total of 46 cases of gallbladder SC/ASC were collected from January 1995 to December 2009, accounting for 4.34% of all gallbladder cancer cases recorded in the same period of time (46/1060). The specimen sources included 16 patients from the Second Xiangya Hospital of Central South University (16/370, 4.32%), 14 from Xiangya Hospital (18/325, 4.31%), 5 from the Third Xiangya Hospital (5/110, 4.55%), 4 from the Hunan Provincial Tumor Hospital (4/100, 4.00%), 5 from the Hunan Provincial People's Hospital (5/105, 4.76%), and 1 from each of the Central Hospital of Changde and the Central Hospital of Loudi (2/50, 4.00%). Among the 46 cases of SC/ASC, 19 patients were males (41.3%) and 27 patients were females (58.7%). These subjects showed an age range of 35-82 years, with a mean of 55.8 ± 9.6 years, including 3 patients ⩽ 45 years of age (6.5%) and 43 patients > 45 years of age (93.5%).

The pathological types included 26 cases of SC (56.5%) and 20 cases of ASC (43.5%). The differentiation among the subjects (judged by the differentiation of SC) included 16 cases of highly differentiated SC (34.8%), 24 cases of moderately differentiated SC (52.2%), and 6 cases of poorly differentiated SC (13.0%). The maximal diameter of the mass was ⩽ 3 cm in 20 cases (43.5%) and > 3 cm in 26 cases (56.5%). Gallbladder stones were found in 28 cases (60.9%); regional lymph node metastasis was confirmed through intraoperative and (or) pathologic examination in 29 cases (63.0%) and tumor invasion of the surrounding tissues and organs outside the gallbladder was intraoperatively observed in 30 cases (65.2%). The TNM staging included 5 cases in stage Ⅰ (10.9%), 7 in stage Ⅱ (15.2%), 17 in stage Ⅲ (43.5%) and 14 in stage Ⅳ (30.4%). The applied surgical procedures comprised 14 cases of radical resection (30.4%), 18 cases of palliative resection (39.1%) and 14 cases of biopsy alone with no tumor excision (30.4%).

Additionally, surgical specimens from 80 cases of gallbladder AC were collected from the Second Xiangya Hospital and the Central Hospital of Loudi from January 2000 to December 2009 for a comparative analysis. Among the 80 cases of AC, 26 patients were males (32.5%) and 54 patients were females (67.5%). The ages of the subjects ranged from 33-80 years, with

a mean of 53.8 ± 9.9 years, including 16 subjects of ≤ 45 years (20.0%) and 64 subjects of > 45 years (80.0%).

The differentiation among this group included 27 cases of highly differentiated carcinoma (33.8%), 25 cases of moderately differentiated carcinoma (33.1%), and 28 cases of poorly differentiated carcinoma (35.0%). The maximal diameter of the mass was ≤ 3 cm in 50 cases (62.5%) and > 3 cm in 30 cases (37.5%). Gallbladder stones were found in 38 cases (47.5%); regional lymph node metastasis was confirmed through intraoperative and (or) pathologic examination in 50 cases (62.5%); tumor invasion of surrounding tissues and organs outside the gallbladder was intraoperatively observed in 49 cases (61.3%). The TNM staging included 8 cases in stage Ⅰ (10%), 13 in stage Ⅱ (16.3%), 38 in stage Ⅲ (47.5%) and 21 in stage Ⅳ (26.3%). The applied surgical procedures comprised 26 cases of radical resection (32.5%), 28 cases of palliative resection (35.0%) and 26 cases of biopsy only with no tumor excision (32.5%).

Through mail or telephone interviews, follow-up information was obtained for the 46 cases of SC/ASC and the 80 cases of AC patients, over a follow-up period of 2 years. Among the 46 SC/ASC patients, postoperative survival was ≥ 1 year in 13 cases (4 patients survived over 2 years) and < 1 year in 33 cases, with an average survival time of 10.07 ± 0.78 mo. Among the 80 AC patients, postoperative survival was ≥ 1 year in 23 cases (9 patients survived over 2 years) and < 1 year in 57 cases, with an average survival time of 10.34 ± 0.63 mo. The above gallbladder SC/ASC and AC surgical resection specimens were fixed in 4% formalin for 24 h to prepare conventional paraffin-embedded sections, at a slice thickness of 4 μm.

Main reagents

Rabbit anti-human ROR2 and WNT5a polyclonal antibodies were purchased from Abgent Company (San Diego, CA, United States). The EnVision™ Detection Kit was obtained from Dao Laboratories (Carpinteria, CA, United States).

Methods

The EnVision immunohistochemical method was applied for ROR2 and WNT5a staining, in strict accordance with the operation manual for the reagents. The main procedures were as follows: the slices were dewaxed and washed → treated with 3% H_2O_2 in methanol for 10 min → treated with trypsin for 15 min → treated with the primary antibody added dropwise, with incubation at 37 ℃ for 60 min → treated with solution A added dropwise, with incubation at 37 ℃ for 30 min → developed with the chromogenic regent for 15 min → lightly stained with hematoxylin for 1 min → dehydrated and transparentized, followed by mounting with neutral resin. Brown particles in the cytoplasm indicated ROR2- and WNT5a-positive cells. The rate of positive cells was observed by examining 400 tumor cells in 10 random fields of a section under a microscope at a high magnification. A patient showing an average rate of positive cells ≥ 25% was considered a positive case, while an average rate < 25% was considered a negative case[22-24]. A positive section provided by Beijing Zhongshan Biotechnology Corp (Beijing, China) was used as the positive control, while replacement of the primary antibody with 5% fetal bovine serum was employed as the negative control.

Statistical analysis

All of the experimental data were input into the SPSS13.0 statistical software package (IBM Corp, Armonk, NY, United States). The relationships between the expression of ROR2 and WNT5a and the histological and clinical factors were investigated with the χ^2 test or Fisher's exact test. The Kaplan-Meier method was applied for univariate survival analysis and log-rank testing. The Cox proportional risk model was utilized to perform multivariate analysis and to determine the 95%CI with the normal approximation test (Wald's test). A probability level of $P < 0.05$ was considered significant.

RESULTS

Comparison of the clinicopathological characteristics of gallbladder SC/ASC and AC with the expression of ROR2 and WNT5a

As shown in Table 1, the proportion of poorly differentiated adenocarcinomas among the AC patients aged > 45 years was significantly higher than among the SC/ASC patients ($P < 0.05$), while the proportion of tumors with a maximal diameter > 3 cm among the patients in the SC/ASC group was significantly higher than in the AC group ($P < 0.05$). The sex, existence of gallstones, TNM stage, occurrence of lymph node metastasis, invasion of the surrounding tissues and organs, applied surgical procedure, and the average survival of the SC/ASC patients showed no significant difference compared with the AC patients ($P > 0.05$).

The Brain-derived neurotrophic factor and bone morphogenetic protein receptor type 1A immunohistochemical reaction products were mainly located in the cytoplasm, with occasional nuclear staining observed (Figures 1 and 2). Among the 46 cases of SC/ASC, ROR2 and WNT5a were positively expressed in 26 cases (56.5%) and 29 cases (63.0%), respectively (judged by positive expression of SC, while a case showing positive expression of AC and negative expression of SC was considered to exhibit negative expression). Among the 80 AC cases, ROR2 and WNT5a were positively expressed in 51 (63.8%) and 49 (61.3%) cases. The positive expression rates of ROR2 and WNT5a among the SC/ASC patients showed no significant difference compared with the AC

Table 1 Comparison of the clinicopathologic characteristics of gallbladder squamous/adenosquamous carcinomas and adenocarcinomas with the expression of ROR2 and WNT5a *n* (%)

Clinicopathologic characteristic	SC/ASC, *n* = 46	AC, *n* = 80	χ^2	*P* value
Sex				
Male	19 (41.3)	26 (32.5)	0.986	0.352
Female	27 (58.7)	54 (67.5)		
Age, yr				
≤ 45	3 (6.5)	16 (20.0)	4.143	0.042
> 45	43 (93.5)	64 (80.0)		
Degree of differentiation				
High	16 (34.8)	27 (33.8)	8.515	0.014
Moderate	24 (52.2)	25 (31.3)		
Poor	6 (13.0)	28 (35.0)		
Maximal diameter of the mass, cm				
≤ 3	20 (43.5)	50 (62.5)	4.280	0.039
> 3	26 (56.5)	30 (37.5)		
Gallstones				
No	18 (39.1)	42 (52.5)	2.093	0.148
Yes	28 (60.9)	38 (47.5)		
TNM stage				
Ⅰ + Ⅱ	12 (26.1)	21 (26.3)	0.287	0.866
Ⅲ	20 (33.5)	38 (47.5)		
Ⅳ	14 (30.4)	21 (26.3)		
Lymph node metastasis				
No	17 (37.0)	30 (37.5)	0.004	0.952
Yes	29 (63.0)	50 (62.5)		
Invasion of the surrounding tissue				
No	16 (34.8)	31 (38.8)	0.197	0.658
Yes	30 (62.5)	49 (61.3)		
Surgical procedure				
Radical	14 (30.4)	26 (32.5)	0.215	0.898
Palliative	18 (39.1)	28 (35.0)		
Unresected	14 (30.4)	26 (32.5)		
Average survival time	10.07 (4-25)	10.34 (3-27)	0.014	0.906
ROR2				
-	20 (43.5)	29 (36.2)	0.642	0.386
+	26 (56.5)	51 (63.8)		
WNT5a				
-	17 (37.0)	31 (38.7)	0.040	0.858
+	29 (63.0)	49 (61.3)		

AC: Adenocarcinoma; SC/ASC: Squamous/adenosquamous carcinoma.

patients ($P > 0.05$).

Relationship of ROR2 and WNT5a expression with the clinicopathological characteristics of gallbladder SC/ASC

The positive expression rates of ROR2 and WNT5a in the patients showing a maximal diameter of the mass ≤ 3 cm, a TNM stage of Ⅰ + Ⅱ, no lymph node metastasis, no invasion in the surrounding tissues and organs, and radical resection were significantly lower than in the patients with a maximal diameter of the mass > 3 cm, a TNM stage of Ⅳ, lymph node metastasis, invasion in the surrounding tissues and organs, and no resection ($P < 0.05$ or $P < 0.01$). The positive expression rate of WNT5a in the patients with highly differentiated SC/ASC was significantly lower than in the patients with poorly differentiated SC/ASC ($P < 0.05$). The expression of ROR2 and WNT5a was not significantly related to the sex, age, pathological type, or existence of gallstones among the patients ($P > 0.05$). The detailed data are shown in Table 2.

Table 2 Relationship of ROR2 and WNT5a expression with the clinicopathologic characteristics of gallbladder squamous/adenosquamous carcinoma *n* (%)

Pathologic characteristic	Number of cases	ROR2			WNT5a		
		Positive cases	χ^2	*P* value	Positive cases	χ^2	*P* value
Pathological type							
Squamous carcinoma	26	16 (61.5)	0.612	0.434	17 (65.4)	0.141	0.708
Adenosquamous carcinoma	20	10 (50.0)			12 (60.0)		
Differentiation							
High	16	5 (31.3)	9.123	0.010	8 (50.0)	1.827	0.401
Moderate	24	15 (62.5)			17 (70.8)		
Poor	6	6 (100.0)			4 (66.7)		
Maximal diameter of the mass in cm							
≤ 3	20	7 (35.0)	6.669	0.010	9 (45.0)	4.945	0.026
≥ 3	26	19 (73.1)			20 (76.9)		
Gallstones							
No	18	11 (61.1)	0.253	0.615	12 (66.7)	0.167	0.683
Yes	28	15 (53.6)			17 (60.7)		
TNM stage							
Ⅰ + Ⅱ	12	4 (33.3)			4 (33.3)		
Ⅲ	20	10 (50.0)	7.824	0.023	14 (70.0)	6.411	0.041
Ⅳ	14	12 (85.7)			11 (78.6)		
Lymph node metastasis							
No	17	5 (29.4)	8.065	0.005	7 (41.2)	6.720	0.010
Yes	29	21 (72.4)			22 (75.9)		
Invasion of the surrounding tissue							
No	16	5 (31.3)	6.376	0.011	7 (43.8)	3.920	0.048
Yes	30	21 (70.0)			22 (73.3)		
Surgical procedure							
Radical	14	4 (28.6)	7.374	0.022	5 (35.7)	77.677	0.019
Palliative	18	11 (61.1)			12 (66.7)		
Unresected	14	11 (78.6)			12 (85.7)		

Relationship of ROR2 and WNT5a expression with the clinicopathological characteristics of gallbladder AC

The positive expression rates of ROR2 and WNT5a in the patients with high differentiation, a maximal diameter of the mass ≤ 3 cm, a TNM stage of Ⅰ + Ⅱ, no lymph node metastasis, no invasion in the surrounding tissues and organs, and radical resection were significantly lower than in the patients with low differentiation, a maximal diameter of the mass > 3 cm, a TNM stage of Ⅳ, lymph node metastasis, invasion in the surrounding tissues and organs, and no resection ($P < 0.05$ or $P < 0.01$). The expression of ROR2 and WNT5a was not significantly related to the sex, age, or existence of gallstones in the AC patients ($P > 0.05$). The detailed data are shown in Table 3.

Relationship of the clinicopathological parameters and WNT5a, ROR2 expression with the average survival of SC/ASC patients

Through mail or telephone interviews, follow-up information was obtained for 46 of the SC/ASC

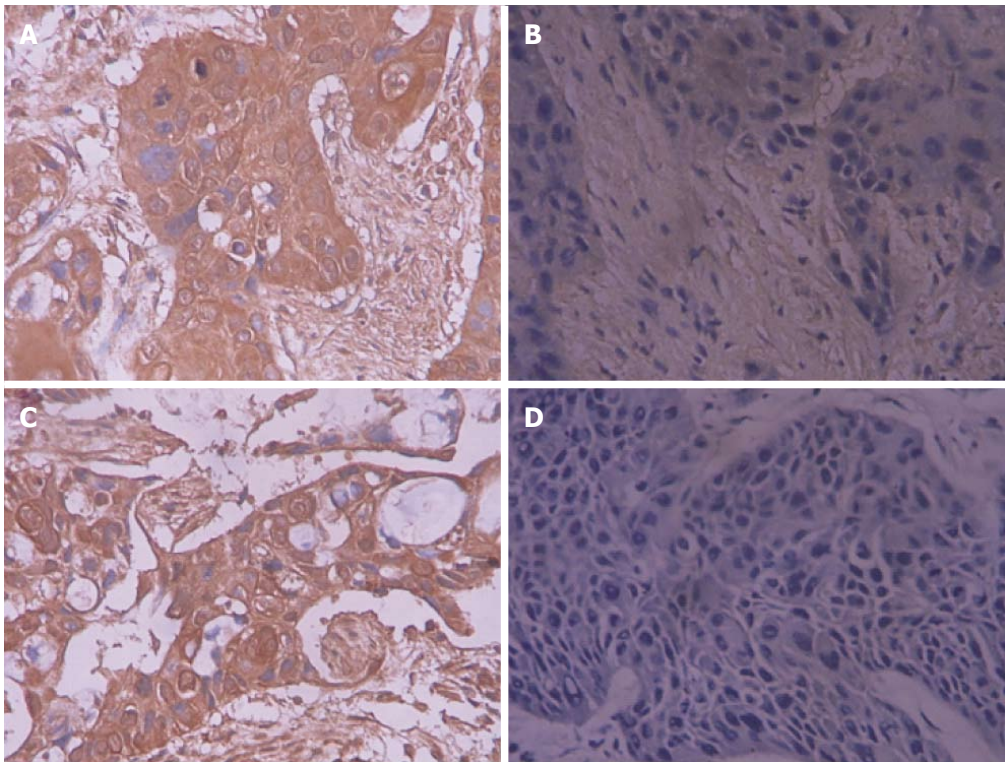

Figure 1 EnVision immunohistochemical staining of squamous carcinoma and adenosquamous carcinoma. A: Positive expression of ROR2 in moderately differentiated squamous carcinoma (SC); B: Negative expression of ROR2 in moderately differentiated SC; C: Positive expression of WNT5a in moderately differentiated adenosquamous carcinoma (ASC); D: Negative expression of WNT5a in poorly differentiated ASC. Microscopic view at × 200.

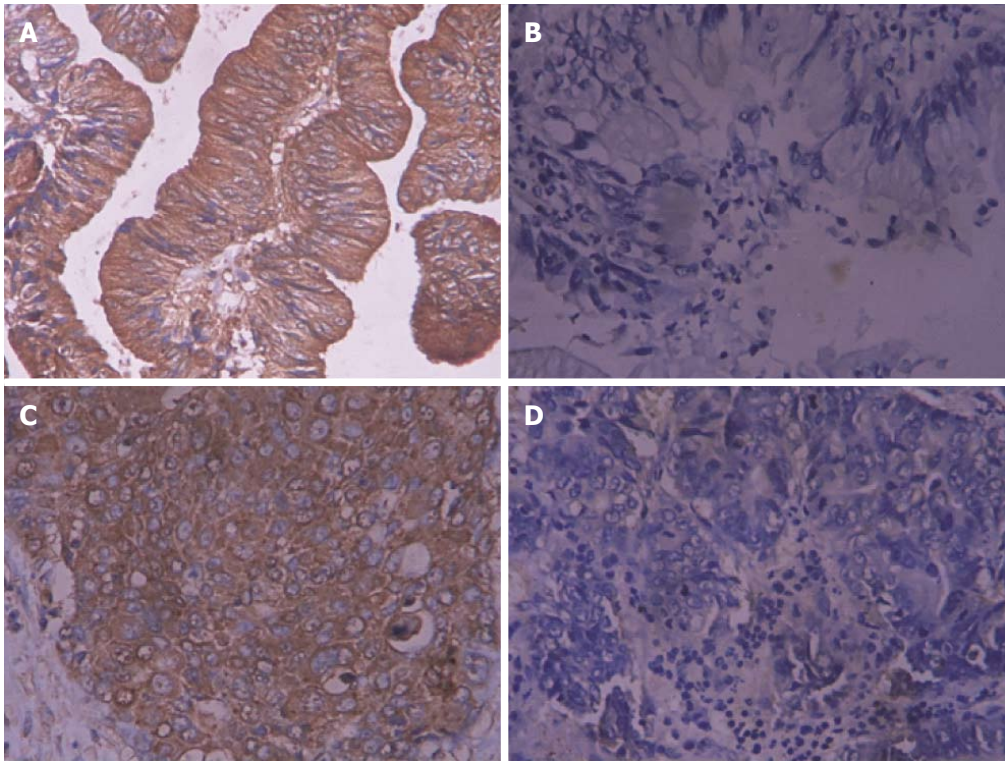

Figure 2 EnVision immunohistochemical staining for adenocarcinoma. A: Positive expression of ROR2 in highly differentiated adenocarcinoma (AC); B: Negative expression of ROR2 in highly differentiated AC; C: Positive expression of WNT5a in highly differentiated AC; D: Negative expression of WNT5a in poorly differentiated AC. A and C: × 200; B and D: × 400.

Table 3 Relationship of ROR2 and WNT5a expression with the clinicopathologic characteristics of gallbladder adenocarcinomas *n* (%)

Pathologic characteristic	Number of cases	ROR2			WNT5a		
		Positive cases	χ^2	*P* value	Positive cases	χ^2	*P* value
Differentiation							
High	27	12 (44.0)	10.352	0.002	11 (40.7)	8.404	0.015
Moderate	25	15 (60.0)			16 (64.0)		
Poor	28	24 (85.7)			22 (78.6)		
Maximal diameter of the mass in cm							
≤ 3	50	26 (52.0)	7.966	0.004	26 (52.0)	4.807	0.029
> 3	30	25 (83.3)			23 (76.7)		
Gallstones							
No	42	26 (61.9)	0.13	0.718	26 (61.9)	0.016	0.899
Yes	38	25 (65.8)			23 (60.5)		
TNM stage							
Ⅰ + Ⅱ	21	7 (33.3)			7 (33.3)		
Ⅲ	38	25 (65.8)	14.968	0	24 (63.2)	12.248	0.002
Ⅳ	21	19 (90.5)			18 (85.7)		
Lymph node metastasis							
No	30	12 (40.0)	11.716	0.001	12 (40.0)	9.132	0.003
Yes	50	39 (78.0)			37 (74.0)		
Invasion of the surrounding tissue							
No	31	13 (41.9)	10.422	0.002	12 (38.7)	10.834	0.001
Yes	49	38 (77.6)			37 (75.5)		
Surgical procedure							
Radical	26	10 (38.5)	12.296	0.002	10 (38.5)	11.68	0.003
Palliative	28	19 (67.9)			17 (60.7)		
Unresected	26	22 (84.6)			22 (84.6)		

patients, over a follow-up period of 2 years. The 2-year survivors were included in the statistical analysis as censored cases. Among the 46 SC/ASC patients, postoperative survival was ≥ 1 year in 13 cases (4 patients survived for over 2 years) and < 1 year in 33 cases, with an average survival time of 10.07 ± 0.78 mo. The results of the Kaplan-Meier survival analysis showed that the degree of differentiation, maximal diameter of the mass, TNM stage, lymph node metastasis, invasion of the surrounding tissues, and applied surgical procedure were closely related to the average survival of the patients with gallbladder SC/ASC ($P < 0.05$ or $P < 0.01$). The survival of the patients with positive expression of ROR2 and WNT5a was significantly shorter compared with the patients with negative expression ($P = 0.000$, $P = 0.001$), as shown in Table 4 and in the survival curves in Figure 3.

The Cox multivariate analysis showed that poor differentiation, a maximal diameter of the mass ≥ 3 cm, a TNM stage of Ⅲ or Ⅳ, the occurrence of lymph node metastasis, invasion of the surrounding tissues and organs, and unresected surgery were negatively correlated with the postoperative survival rate and positively correlated with mortality, which are risk factors and independent prognostic predictors. Positive expression of ROR2 or WNT5a was negatively correlated with the postoperative survival rate and positively correlated with mortality, which are the risk factors and independent prognostic predictors (Table 5).

Table 4 Relationship of squamous/adenosquamous carcinoma clinicopathological parameters and ROR2 and WNT5a expression with average survival

Grouping	Number of cases, *n*	Average survival in mo	χ^2	*P* value
Sex				
Male	19	10.74 (6-24)	0.767	0.381
Female	27	9.85 (4-24)		
Age, yr				
≤ 45	3	15.67 (8-24)	2.023	0.155
> 45	43	9.84 (4-25)		
Pathological type				
Squamous carcinoma	26	10.19 (4-24)	0.223	0.637
Adenosquamous carcinoma	20	10.25 (4-24)		
Degree of differentiation				
High	16	13.81 (5-24)	19.125	0.000
Moderate	24	8.92 (4-18)		
Poor	6	5.83 (4-9)		
Maximal diameter of the mass, cm				
≤ 3	20	14.35 (7-24)	31.337	0.000
> 3	26	7.04 (4-11)		
Gallstones				
No	18	8.22 (4-12)	3.730	0.053
Yes	28	11.50 (4-24)		
TNM stage				
Ⅰ + Ⅱ	12	17.00 (9-24)	51.139	0.000
Ⅲ	20	9.20 (7-15)		
Ⅳ	14	5.86 (4-8)		
Lymph node metastasis				
No	17	14.24 (4-24)	16.219	0.000
Yes	29	7.86 (4-15)		
Invasion of the surrounding tissue				
No	16	15.75 (9-24)	32.271	0.000
Yes	30	7.27 (4-12)		
Surgical procedure				
Radical	14	16.64 (10-24)	50.165	0.000
Palliative	18	8.50 (6-12)		
Unresected	14	6.00 (4-8)		
ROR2				
-	20	13.65 (6-24)	16.502	0.000
+	26	7.58 (4-15)		
WNT5a				
-	17	13.77 (6-24)	10.844	0.001
+	29	8.14 (4-15)		

Relationship of the clinicopathological parameters and WNT5a, ROR2 expression with the average survival of AC patients

Through mail or telephone interviews, follow-up information was obtained for 80 of the SC/ASC patients, over a follow-up period of 2 years. The 2-year survivors were included in the statistical analysis as censored cases. Among the 80 AC patients, postoperative survival was ≥ 1 year in 23 cases (9 patients survived for over 2 years) and < 1 year in 57 cases, with an average survival time of 10.34 ± 0.63 mo. The results of the Kaplan-Meier survival analysis showed that the degree of differentiation, maximal diameter of the mass, TNM stage, lymph node metastasis, invasion of the surrounding tissues, and applied surgical procedure were closely related to the average survival of the patients with gallbladder AC ($P = 0.000$). Survival

Table 5 Multivariate Cox regression analysis for survival of patients with gallbladder squamous/adenosquamous carcinoma

Grouping	Factor	B	SE	Wald	*P* value	RR	95%CI	
							Lower	Upper
Pathological type	SC/ASC	0.094	0.346	0.074	0.786	1.099	0.558	2.164
Degree of differentiation	High/Moderate/Poor	0.833	0.369	5.096	0.024	2.300	1.116	4.741
Maximal diameter of the mass, cm	≤ 3/> 3	2.374	0.743	10.209	0.001	10.740	2.504	46.075
Gallstones	No/Yes	0.641	0.420	2.329	0.127	1.898	0.833	4.324
TNM stage	Ⅰ + Ⅱ/Ⅲ/Ⅳ	1.362	0.476	8.187	0.004	3.904	1.536	9.924
Lymph node metastasis	No/Yes	1.792	0.564	10.095	0.001	6.011	1.987	18.128
Invasion of the surrounding tissue	No/Yes	2.648	0.782	11.466	0.001	14.126	3.050	65.413
Surgical procedure	Radical/Palliative/Unresected	1.104	0.481	5.268	0.022	3.016	1.175	7.743
ROR2	-/+	1.623	0.674	5.799	0.016	5.068	1.353	18.992
WNT5a	-/+	1.231	0.489	6.337	0.012	3.425	1.313	8.930

SC/ASC: Squamous/adenosquamous carcinoma.

Table 6 Relationship of the adenocarcinomas' clinicopathological parameters and ROR2 and WNT5a expression with average survival

Grouping	Number of cases, *n*	Average survival in mo	χ^2	*P* value
Sex				
Male	26	9.58 (3-24)	2.567	0.109
Female	54	11.30 (3-24)		
Age, yr				
≤ 45	16	10.81 (4-24)	0.003	0.956
> 45	64	10.72 (3-24)		
Degree of differentiation				
High	27	15.07 (5-24)	32.501	0.000
Moderate	25	10.60 (4-24)		
Poor	28	6.68 (3-14)		
Maximal diameter of the mass, cm				
≤ 3	50	13.70 (6-24)	68.283	0.000
> 3	30	5.80 (3-10)		
Gallstones				
No	42	10.19 (3-24)	0.246	0.620
Yes	38	11.34 (4-24)		
TNM stage				
Ⅰ + Ⅱ	21	18.96 (5-24)	105.825	0.000
Ⅲ	38	9.29 (6-15)		
Ⅳ	21	5.14 (3-7)		
Lymph node metastasis				
No	30	16.27 (4-24)	42.372	0.000
Yes	50	7.42 (3-14)		
Invasion of the surrounding tissue				
No	31	16.68 (7-24)	55.535	0.000
Yes	49	6.98 (3-11)		
Surgical procedure				
Radical	26	18.31 (10-24)	113.141	0.000
Palliative	28	8.64 (6-11)		
Unresected	26	5.42 (3-9)		
ROR2				
-	29	15.93 (6-24)	32.994	0.000
+	51	7.78 (3-19)		
WNT5a				
-	31	15.48 (4-24)	31.654	0.002
+	49	7.74 (3-18)		

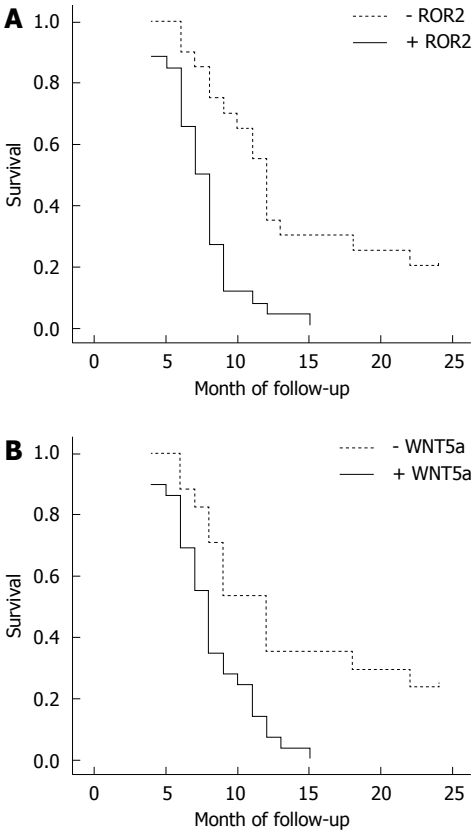

Figure 3 Survival curves for squamous/adenosquamous carcinoma. A: Positive and negative expression of ROR2 in squamous/adenosquamous carcinoma (SC/ASC); B: Positive and negative expression of WNT5a in SC/ASC.

of the patients with positive expression of ROR2 and WNT5a was significantly shorter compared with the patients with negative expression (P = 0.000), as shown in Table 6 and in the survival curves in Figure 4.

The Cox multivariate analysis showed that poor differentiation, a maximal diameter of the mass ≥ 3 cm, a TNM stage of Ⅲ or Ⅳ, the occurrence of lymph node metastasis, the invasion of the surrounding tissues and organs, and unresected surgery were negatively correlated with the postoperative survival rate and positively correlated with mortality, which are risk factors and independent prognostic predictors. The positive expression of ROR2 or WNT5a was negatively correlated with the postoperative survival rate and positively correlated with mortality, which are risk factors and independent prognostic predictors (Table 7).

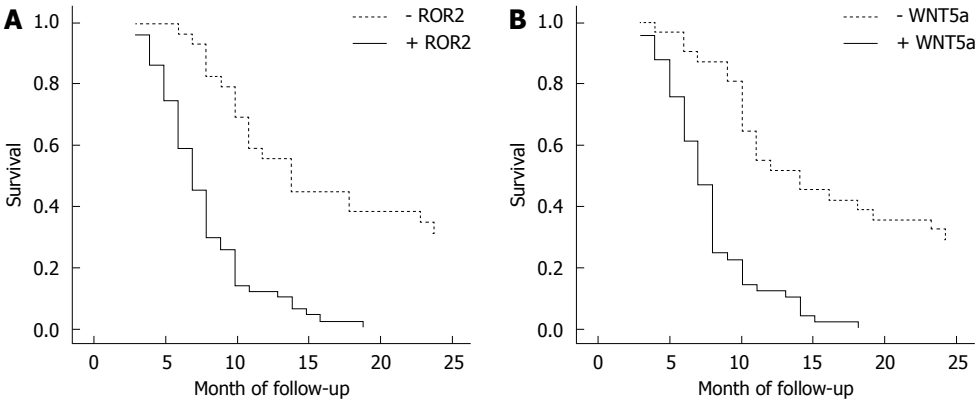

Figure 4 Survival curves for adenocarcinoma. A: Positive and negative expression of ROR2 in AC; B: Positive and negative expression of WNT5a in AC.

Table 7 Multivariate Cox regression analysis for survival of patients with gallbladder adenocarcinomas

Grouping	Factor	B	SE	Wald	*P* value	RR	95%CI	
							Lower	Upper
Degree of differentiation	High/Moderate/Poor	1.097	0.496	4.982	0.027	2.995	1.133	7.918
Maximal diameter of the mass, cm	≤ 3/> 3	0.985	0.463	4.526	0.033	2.678	1.081	6.636
Gallstones	No/Yes	0.412	0.381	1.169	0.280	1.510	0.716	3.186
TNM stage	Ⅰ + Ⅱ/Ⅲ/Ⅳ	1.396	0.459	9.250	0.002	4.039	1.643	9.931
Lymph node metastasis	No/Yes	1.351	0.465	8.441	0.004	3.861	1.552	9.606
Invasion of the surrounding tissue	No/Yes	1.644	0.583	7.952	0.005	5.176	1.651	16.227
Surgical procedure	Radical/Palliative/ Unresected	1.737	0.531	10.701	0.001	5.680	2.006	16.083
ROR2	-/+	2.023	0.674	9.009	0.003	7.561	2.018	28.333
WNT5a	-/+	1.931	0.649	8.853	0.003	6.896	1.933	24.607

DISCUSSION

Gallbladder cancer is not common in the digestive system, and AC accounts for > 85% of all gallbladder cancers. Most ACs are highly or moderately differentiated, with few poorly differentiated cases being observed[22]. SC and ASC are rare subtypes of gallbladder cancer, showing a combined incidence rate of 1.4%-10.4% among all gallbladder cancers. Of the 1060 cases of gallbladder cancer examined in this study, 46 (4.34%) were SC/ASC, which is consistent with the data reported in most of the literature[1,2].

SC/ASC shares similar clinical manifestations with AC, including an insidious onset and lack of specific clinical manifestations in the early stage, or only symptoms of chronic cholecystitis; hence, early diagnosis is very difficult. If a patient experiences persistent upper abdominal pain, mass and jaundice, the disease has progressed to the late stage, with abnormal results being obtained in various examinations[1-6,22]. The data evaluated in this study showed that 73.8% the SC/ASC cases were in TNM stage Ⅲ or Ⅳ, similar to the percentage for AC (73.8%). According to most of the available literature, the proliferation of SC is stronger than that of AC, associated with high malignancy. The prognosis of SC/ASC is poor compared with AC, but its metastasis potential is low. Therefore, most gallbladder SCs manifest as a giant lump and are prone to direct invasion of the surrounding organs, with little metastasis being observed in the lymph nodes and distant organs[1-6,25].

The data from this study showed that the proportion of SC/ASC cases displaying a maximal diameter of the mass > 3 cm (56.5%) was significantly higher than for the AC cases (37.5%), while the incidence of lymph node metastasis and invasion of the surrounding tissues and organs was not significantly different between these groups. The prognosis of gallbladder SC/ASC and AC was very poor. All of the patients of TNM stage of Ⅲ or above died within 2.5 years after surgery, with 5-year survival rates of less than 4% being observed, while the 5-year survival of the patients presenting TNM stage Ⅰ was > 60%. Therefore, early diagnosis is particularly important.

The data from this study showed that the average postoperative survival of the SC/ASC patients was 10.07 ± 0.78 mo, similar to that of the AC patients (10.34 ± 0.63 mo). Only 4 SC/ASC patients and 9 AC patients in TNM stage Ⅰ + Ⅱ survived for over 2 years, demonstrating the poor prognosis of both SC/ASC and AC. Similar to AC, the main treatment for SC/ASC is surgery, but the rate of radical resection is low[1-6,25]. In this study, the radical resection rate among the 46 SC/ASC cases was 30.4%, similar to that for AC (32.5%). The survival time of the patients in the radical

resection group was significantly longer than in the palliative resection group and the no resection group; therefore, extended radical surgery may improve the prognosis of the patients.

SC/ASC and AC are not sensitive to chemotherapy, but radiation therapy may have an effect on SC/ASC patients, as reported in the literature, though determination of the specific outcome awaits further observations and the accumulation of relevant data[1-6]. In summary, the results of this study suggest that the clinical manifestations, biological behavior, treatment and postoperative prognosis of SC/ASC are similar to those of AC, with no obvious differences being observed.

The ROR family of receptors belongs to a class of orphan receptors among the receptor tyrosine kinases, which are highly evolutionarily conserved. In mammals, the ROR family consists of two structurally related proteins, ROR1 and ROR2. ROR2 plays an important role in the development of the nervous system and limbs. It has been reported that ROR2 can bind to Wnt5a, CKI and other factors involved in the regulation of canonical and non-canonical Wnt signaling pathways, but the relationship between ROR2 and tumor cell migration remains unclear[24].

The structure of the ROR2 protein includes three important domains: a cytoplasmic domain, a transmembrane domain and an extracellular domain[25]; it has also been shown that ROR2 exhibits a Wnt receptor domain-like structure[26]. Binding of ROR2 with Wnt5a can trigger a signaling pathway mediated by ROR2, and the Wnt5a protein may have an antagonistic effect on the canonical Wnt signaling pathway via this pathway. However, when the receptors of Frizzled4 and LRPS are expressed on the cell surface, the effect of Wnt5a and ROR2 binding is significantly reduced or lost, and the difference in the degree of binding can reflect the activation of the canonical Wnt signal transduction pathway.

The functions of ROR2 and Wnt5a suggest that Wnt5a may play different roles in different tumors. To date, only a few studies examining ROR2 in relation to the occurrence and development of human tumors have been reported. As mentioned above, expression of ROR2 is closely associated with the biological behavior as well as the clinical manifestations of various malignant tumors, including digestive tract cancer[9-11,12], breast cancer[11], and medulloblastoma[13]. Further studies suggested that high expression of ROR2 is often founded in poorly differentiated malignancies in a late clinical stage with strong invasiveness, which is consistent with our results. Therefore, ROR2 is a significant potential biological marker for evaluating the prognosis of the patients with malignant tumors, and high expression of ROR2 may suggest a poor prognosis.

The Wnt5a gene is located on chromosome 3p14.2-p21.1 and encodes a growth factor rich in cysteine, which is involved in signaling transduction between cells during the growth and differentiation of cells[27,28]. Wnt5a can activate both the canonical Wnt/-catenin pathway and the non-canonical Wnt/Ca^{2+} pathway. Additionally, the Wnt/Ca^{2+} pathway can interact with the canonical Wnt pathway to play roles in cell differentiation, maturation and tumor development[22,23]. The main biological function of Wnt5a is related to the development and maturation of normal tissues and organs.

Wnt5a is closely associated with a variety of malignant tumors, but the existing reports indicate that the biological effects of Wnt5a are not consistent in different malignancies. Thus, there is significant disagreement in the understanding of the roles of Wnt5a, and some studies have suggested that Wnt5a presents the characteristics of an oncogene. The existing evidence indicates that sustained expression or over-expression of Wnt5a plays an important role in the onset of cancer, through affecting the proliferation, differentiation, invasion and metastasis of tumor cells. For example, Wnt5a is over-expressed and influences the migration and invasion of tumors in cutaneous melanoma[17], breast cancer cells[18], gastric cancer[19], non-small cell lung cancer[20] and prostate cancer[21], exhibiting the characteristics of an oncogene. Further studies revealed that malignant tumors exhibiting high expression of Wnt5a are often poorly differentiated, in a late clinical stage and prone to metastasis with strong invasiveness. Wnt5a is considered to be an important biological indicator for assessing the prognosis of patients with malignant tumors, and high expression of Wnt5a suggests a poor prognosis.

Wnt5a may display the characteristics of a tumor suppressor gene. It can act as a tumor suppressor to partially reduce or delay the occurrence and metastasis of a malignant tumor. Iozzo *et al*[24] found that Wnt5a was up-regulated in most malignant tumors but was down-regulated in pancreatic cancer, indicating that the roles of Wnt5a in tumors are not fully consistent. Liang *et al*[25] observed that a lack of Wnt5a readily led to the occurrence of human hematopoietic malignancies and that Wnt5a could act as a tumor suppressor to inhibit the proliferation of B cells and tumorigenesis; therefore, Wnt5a is a potential therapeutic target for human acute lymphoblastic leukemia and myeloid leukemia. In addition, Wnt5a plays the role of suppressor in colorectal cancer[14], neuroblastoma[15] and leukemia[16], where its expression is down-regulated. Thus, considerable controversy remains regarding the biological role of Wnt5a in different tumors.

No study addressing the expression levels of ROR2 and Wnt5a in gallbladder SC/ASC and AC tissues has previously been reported in the literature. The results of the present study showed that the positive expression rates of ROR2 and WNT5a were not significantly different between SC/ASC and AC tissues ($P > 0.05$). While the positive expression rates of Wnt were not significantly different between highly differentiated SC/AS and poorly differentiated SC/AS

($P > 0.05$), the positive expression rates of ROR2 and WNT5a in the SC/ASC or AC patients with high differentiation, a maximal diameter of the mass ⩽ 3 cm, a TNM stage of Ⅰ + Ⅱ, no lymph node metastasis, no invasion in the surrounding tissues and organs, and radical resection were significantly lower than in the patients with a maximal diameter of the mass > 3 cm, a TNM stage of Ⅳ, lymph node metastasis, invasion in the surrounding tissues and organs, and no resection ($P < 0.05$ or $P < 0.01$).

The results of the Kaplan-Meier survival analysis showed that the degree of differentiation, maximal diameter of the mass, TNM stage, lymph node metastasis, invasion of the surrounding tissues, and applied surgical procedure were closely related to the average survival of the patients with gallbladder SC/ASC or AC ($P < 0.05$ or $P < 0.01$). Survival of the patients presenting positive expression of ROR2 and WNT5a was significantly shorter compared with the patients presenting negative expression results ($P < 0.01$). Cox multivariate analysis revealed that poor differentiation, a maximal diameter of the mass ⩾ 3 cm, a TNM stage of Ⅲ or Ⅳ, the occurrence of lymph node metastasis, invasion of the surrounding tissues and organs, and unresected surgery were negatively correlated with the postoperative survival rate of the patients and positively correlated with mortality, which are risk factors and independent prognostic predictors.

Our experimental results are consistent with reported findings regarding the expression levels of ROR2 and Wnt5a in other epithelial malignancies from researchers from other countries, suggesting that ROR2 and Wnt5a play an important role in the occurrence, progression, biological behavior and prognosis of gallbladder SC/ASC and AC. Gallbladder SC/ASC and AC cases showing high expression of ROR2 and Wnt5a are highly malignant, exhibit rapid progression, and are prone to regional lymph node metastasis with strong invasiveness. Thus, ROR2 and Wnt5a are both important biological markers reflecting the prognosis of patients with gallbladder SC/ASC and AC. Considering the findings presented in the relevant literature, detection of the expression levels of ROR2 and (or) Wnt5a in benign gallbladder lesions may have important clinical pathological significance in the prevention and early diagnosis of gallbladder cancer. Further research on this topic is awaited.

COMMENTS

Background

Gallbladder carcinoma (GBC) is the most common and aggressive type of carcinoma among the biliary tree cancers. Early diagnosis and radical surgery contribute improved prognosis of GBCs. The diagnosis of GBC mainly depends on non-invasive auxiliary imaging and invasive examination, such as laparoscopy and biopsy. However, ideal biological markers for the diagnosis, prognosis and targeted therapy of GBCs have not been established. In this study, we detected the expression levels of ROR2 and WNT5a in surgical resection specimens from a total of 46 cases of gallbladder squamous/adenosquamous carcinoma (SC/ASC) and 80 cases of gallbladder adenocarcinomas (AC). The results showed that ROR2 and WNT5a expression are negatively correlated with postoperative survival rate and positively correlated with mortality.

Research frontiers

Tumor markers have had an increasing significance in the diagnosis and evaluation of GBC. Previous studies found that CA242, CA15-3, CA19-9 and CA125 are fairly good markers for discriminating patients of carcinoma of the gallbladder from cholelithiasis. Combined CA242 and CA125 detection achieved the best sensitivity and specificity. Serum markers seem to be less sensitive when used individually in carcinomas of the gallbladder but may prove useful in combination. However, the ideal biological markers for the diagnosis, prognosis and targeted therapy of GBCs have not been established.

Innovations and breakthroughs

This is the first report investigating ROR2 and WNT5a expression in clinical samples from two different types of gallbladder cancer (SC/ASC and AC) using the EnVision immunohistochemical staining technique, and reveals their correlation with clinicopathologic characteristics in both types of gallbladder cancer.

Applications

The expression and clinicopathological significance of ROR2 and Wnt5a could be applied to the prevention and early diagnosis of gallbladder cancer in benign gallbladder lesions. The combined detection of ROR2 and WNT5a as biological markers might increase sensitivity for the diagnosis and prognosis of GBC.

Terminology

SC/ASC and AC are two major types of gallbladder cancer with slightly different clinicopathologic characteristics. Squamous carcinoma shows slight differences from adenocarcinoma gallbladder cancer with an advanced T stage but less common nodal involvement and distant metastasis (Kalayarasan *et al*, *Am J Surg* 2013; 206: 380-385).

Peer-review

The paper is significant hence it studies a rare type of tumor.

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ORIGINAL ARTICLE

Observational Study

Serum omentin and vaspin levels in cirrhotic patients with and without portal vein thrombosis

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Abstract

AIM

To investigate serum omentin and vaspin levels in cirrhotic patients; and to assess the relationship of these levels with hemostatic parameters, metabolic abnormalities, cirrhosis severity and etiology.

METHODS

Fifty-one cirrhotic patients (17 with portal vein thrombosis) were analyzed. Serum omentin and vaspin levels were measured with commercially available direct enzyme-linked immunosorbent assays (ELISAs). To assess platelet activity, the following tests were performed using a MULTIPLATE®PLATELET FUNCTION ANALYZER: (1) an ADP-induced platelet activation test; (2) a cyclooxygenase dependent aggregation test (ASPI test); (3) a von Willebrand factor and glycoprotein Ib-dependent aggregation (using ristocetin) test (RISTO test); and (4) a test for thrombin receptor-activating peptide-6 induced activation of the thrombin receptor, which is sensitive to ⅡbⅢa receptor antagonists.

RESULTS

Omentin, but not vaspin, serum concentrations were significantly decreased in patients with portal vein thrombosis (PVT) (P = 0.01). Prothrombin levels were significantly increased in patients with PVT (P = 0.01). The thrombin receptor activating peptide (TRAP) test results were significantly lower in the PVT group (P = 0.03). No significant differences in adipokines serum levels were found regarding the etiology or severity of liver cirrhosis assessed according to the Child-Pugh or Model of End-Stage Liver Disease (MELD) scores. There was a significant increase in the TRAP (P = 0.03), ASPI (P = 0.001) and RISTO high-test (P = 0.02) results in patients with lower MELD scores. Serum omentin and vaspin levels were significantly down-regulated in patients without insulin resistance (P = 0.03, P = 0.02, respectively). A positive relationship between omentin and vaspin levels were found both when all of the patients were analyzed (r = 0.41, P = 0.01) and among those with PVT (r = 0.94, P < 0.001).

CONCLUSION

Serum omentin levels are increased in patients without PVT. Cirrhosis origin and grade do not affect omentin and vaspin levels. The analyzed adipokines do not influence platelet activity.

Key words: Omentin; Vaspin; Cirrhosis; Adipokine; Portal vein thrombosis; Portal hypertension

Core tip: Accumulating data suggest that obesity and insulin resistance are related to a more rapid progression of chronic liver diseases, the development of cirrhosis and its complications. Some adipokines have been suggested to contribute to the complicated pathophysiology of hepatic injury and repair. Ongoing research has revealed alterations in the levels and expression of various adipokines in cirrhosis. Portal vein thrombosis (PVT) has been considered to be a complication of more advanced liver cirrhosis. The data regarding novel adipokines in liver cirrhosis is scare and ambiguous. The current study evaluated the serum concentrations of omentin and vaspin in patients with liver cirrhosis of different origins and stages with and without PVT. The relationships of these measures with disease severity and etiology, platelet activity, hemostatic parameters and potential complications were also assessed. The study included 51 patients with cirrhosis of different etiologies (alcohol in 30 patients, hepatitis C virus infection in 15, autoimmune hepatitis in 6). Seventeen these patients manifested portal vein thrombosis confirmed by contrast-enhanced computed tomography.

KuklaM, Waluga M, Żorniak M, Berdowska A, Wosiewicz P, Sawczyn T, Bułdak RJ, Ochman M, Ziora K, Krzemiński T, Hartleb M. Serum omentin and vaspin levels in cirrhotic patients with and without portal vein thrombosis. *World J Gastroenterol* 2017; 23(14): 2613-2624 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i14/2613.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i14.2613

INTRODUCTION

Liver cirrhosis is associated with progressive liver impairment, leading to the development of numerous complications, including portal hypertension and portal vein thrombosis (PVT)[1]. PVT is generally recognized as rare in the general population, being primarily a consequence of myeloproliferative diseases, genetic or acquired thrombophilia or inflammation in the abdominal cavity[2]. However, PVT is a relatively common complication of liver cirrhosis, with an estimated frequency of 5%-28%[3,4]. Excessive hepatic deposition of fibrotic tissue contributes to intra-hepatic resistance, an up-regulation of portal blood pressure and a reduction in portal blood flow into the liver[5].

Adipokines are polypeptide hormones that are primarily produced by adipocytes. Apart from fat cells, adipose tissue is composed of stromal cells, including macrophages, fibroblasts and infiltrating monocytes, all of which may serve as an additional source of adipokines[6]. Accumulating data reveal disturbances in the secretion of some adipokines in chronic liver diseases (CLDs) and cirrhosis, leading to the complex pathophysiology of hepatic injury and healing[7]. Some adipokines are also recognized to be active as pro-fibrotic and pro-thrombotic agents. The best of these is known leptin, receptors for which have been identified in hepatic stellate cells and many types of vascular cells, including sinusoidal endothelial cells (SECs), macrophages (Kupffer cells) and platelets[8-10]. Another

adipokine, adiponectin, exerts opposite anti-fibrotic and anti-inflammatory effects[11]. Novel adipokines and hepatokines were recently described, and their role in liver diseases are now being intensively investigated[7].

Omentin-1 (intelectin-1, also known as an active endothelial factor) is a newly identified adipokine that is highly and selectively expressed in visceral adipose tissue[12,13]. The data regarding hepatic omentin expression is equivocal. Our previous study confirmed omentin expression in the liver tissue from chronic hepatitis C (CHC) patients[14]. However, an earlier study by Yang *et al*[15] found higher omentin expression in the stroma-vascular cells of omental fat and lower expression in the intestine and lung; no expression was observed in the liver, kidney and pancreas. Omentin has been suggested to be a "good adipokine" because its serum concentration were negatively associated with a multiplicity of metabolic risk factors in metabolic syndrome (MS)[16]. A previous study by Pan *et al*[17] revealed significantly lower serum omentin levels in patients with diabetes mellitus (DM). However, the role of omentin in CLD is unclear. A small number of studies have shown that omentin serum are levels increased in CHC, NAFLD and cirrhosis[14,18,19].

Vaspin (visceral adipose tissue-derived serine protease inhibitor) is a novel adipokine that was isolated from both the visceral and subcutaneous white adipose tissues of subjects with obesity and impaired glucose tolerance[20]. Vaspin has also been confirmed to down-regulate the expression of profibrogenic and proinflammatory agents, such as leptin, tumor necrosis factor (TNF)-alpha and resistin[7]. Some studies have indicated that the induction of vaspin mRNA expression in human adipose tissue might be a compensatory mechanism associated with obesity and increasing insulin resistance (IR)[7]. Serum levels of vaspin in CHC patients without fibrosis or with less advanced fibrosis were significantly lower than in healthy controls. However, in patients with bridging fibrosis or cirrhosis, the levels were almost as high as in the control group[21,22]. In NAFLD patients, vaspin serum levels seemed to be higher in patients with definite NASH and more advanced fibrosis[23,24].

In light of the aforementioned studies, we decided to investigate serum concentrations of two novel adipokines, omentin and vaspin, in cirrhotic patients with and without PVT. These analyses were performed to assess their prothrombotic action. We then analyzed the association between the serum levels of these adipokines and hemostatic parameters, platelet counts and platelet-aggregation activity markers. We also elucidated the relationship between omentin and vaspin serum levels and cirrhosis severity, etiology and metabolic abnormalities.

MATERIALS AND METHODS

Study population

A total of 51 patients (16 females) with cirrhosis of different etiologies [alcohol in 30 patients (51%), hepatitis C virus (HCV) infection (genotype 1b) in 15 (29%), and autoimmune hepatitis in 6 (12%)] were enrolled. After meeting the qualification criteria, the presence of portal vein thrombosis in 17 was confirmed using contrast-enhanced computed tomography. Data on complications of the liver disease, present and past co-morbidities and current medication use were collected. Patients were excluded for the following reasons: infection with HCV genotypes other than 1b; hepatitis B virus infection; human immunodeficiency virus (HIV) co-infection; drug abuse; the presence of neoplastic, thyroid or psychiatric diseases; or renal or heart failure. Contrast-enhanced spiral computed-tomography was performed on each patient to confirm the presence of PVT. Computed tomography was independently evaluated by two experienced radiologists. The severity of cirrhosis was evaluated by the Model of End-Stage Liver Disease (MELD) and the Child-Pugh score. For further analysis, the patients were divided according to their Child-Pugh score (from A to C) or divided in two subgroups according to MELD scores > 18 and ⩽ 18. This cut-off was determined based on studies that assessed MELD predictive values in patients with end-stage liver disease[25]. The baseline clinical and laboratory characteristics of the patients are presented in Table 1.

Ethics statement

The study protocol was approved by the Local Bioethical Committee of the Medical University of Silesia (Approval of Committee NoKNW/0022/KB1/45/Ⅱ/15, Nov^{17}2015). All of the clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. All of the study participants provided informed written consent prior to study enrollment.

Biochemical and serological assays

Biochemical parameters were measured using routine methods. The upper limit of ALT activity was set at 38 IU/L, that of AST at 40 IU/L and that of gamma-glutamyltransferase (GGT) activity at 50 IU/L. The upper total serum bilirubin concentration was set at 17 μmol/L. Insulin concentrations were measured with a DiaMetra Insulin EIA Kit, Cat. No DKO076 (DiaMetra, Italy). For IR estimation, the homeostatic model assessment (HOMA-IR) was calculated using the following formula: fasting insulin level (mUI/L) × fasting glucose level (mg/dL)/405. With respect to the HOMA-IR value, the patients were divided into two subgroups - below and equal or greater than 3.0. The cut-off was determined on the basis of reviewed recent literature[26].

The blood samples were drawn from the antebrachial vein after 16 h of fasting and were the centrifuged. The serum was frozen and stored for further analysis at a temperature of -70 ℃. Commercially available direct enzyme-linked immunosorbent assays (ELISA)

Table 1 General characteristics and basic laboratory tests of cirrhotic patients with and without portal vein thrombosis

	All patients *n* = 51	PVT (+) *n* = 17	PVT (-) *n* = 34	*P* value
Sex (M/F), *n* (%)	35/16 (68.6/31.4)	12/5 (70.6/29.4)	23/11 (67.6/32.4)	$P > 0.05$
Age (yr)	52 (26-80)	56 (26-80)	54 (27-68)	$P > 0.05$
BMI (kg/m^2)	27 (17-40)	27 (20-40)	27 (17-36)	$P > 0.05$
Child-Pugh (points)	7.7 (5-12)	7.3 (5-11)	7.9 (5-12)	$P > 0.05$
MELD score (points)	13.9 (6-26)	12.8 (9-22)	14.5 (6-26)	$P > 0.05$
Bilirubin (mg/dL)	3.4 (0.6-14.3)	2.3 (1.1-4.8)	3.9 (0.6-14.3)	$P > 0.05$
Hemoglobin (g/dL)	7.5-15.3	11.9 (8.4-15)	12.2 (7.5-15.3)	$P > 0.05$
Platelets (× 10^3/mm^3)	96 (18-330)	83 (32-149)	103 (18-330)	$P > 0.05$
WBC (× 10^3/mm^3)	5.1 (1.3-15.3)	4.3 (1.9-11.2)	5.5 (1.3-15.3)	$P > 0.05$
Albumin (g/dL)	3.2 (1.7-5.1)	3.2 (2.4-4.3)	3.2 (1.7-5.1)	$P > 0.05$
ALT (U/L)	39 (15-130)	41.6 (15-130)	38.1 (15-115)	$P > 0.05$
AST (U/L)	71.2 (18-216)	69 (18-164)	72.1 (20-216)	$P > 0.05$
GGTP (U/L)	122 (25-489)	180.3 (25-489)[1]	91.2 (13-236)	$P = 0.02$
Cholesterol (mg/dL)	176 (45-409)	147.8 (45-284)	187 (87-409)	$P > 0.05$
HDL-Ch (mg/dL)	46.2 (13-78)	33.6 (17-45)	49.6 (13-78)	$P > 0.05$
LDL-Ch (mg/dL)	97.5 (19-203)	83 (19-171)	102.4 (43-203)	$P > 0.05$
TG (mg/dL)	127 (47-407)	133.3 (47-341)	125.3 (48-407)	$P > 0.05$
Ascites	19/51 (42%)	8/17 (47%)	15/34 (44%)	$P > 0.05$

[1]GGTP level was significantly higher in PVT (+) patients. *P* value < 0.05 was considered statistically significant. The data are shown as the mean and range or percentage where applicable. BMI: Body mass index; TG: Triglycerides.

were used for measurement of the serum omentin and vaspin levels (BioVendor; Brno, Czech Republic).

Blood platelets play a pivotal role in physiological hemostasis but also in the development of thrombosis. In addition to increased circulating prothrombotic agents, such as von Willebrand factor (vWf), changes in platelet biology and function may underlie up-regulated thrombotic risk in cirrhosis. These changes include an increase in mean platelet volume, enhanced platelet aggregatory response to agonists and a resistance to the anti-aggregatory effects of nitric oxide and prostacyclin I_2.

Platelet function testing is used to analyze inherited and acquired platelet function disorders. In our study, platelet activity was examined with the MULTIPLATE® PLATELET FUNCTION ANALYZER (Roche; Basel, Switzerland) using multiple electrode aggregometry (MEA). The Multiplate® analyzer is an easy-to-use instrument that standardizes platelet function testing in small quantities of whole blood.

Platelet activity was examined using a MULTIPLATE® PLATELET FUNCTION ANALYZER (Roche; Basel, Switzerland) and MEA. This method is recommended for conducting studies on platelets by the Clinical and Laboratory Standards Institute (document H58-A, 2008). The following tests were performed: ADP-induced platelet activation - ADP test; Cyclooxygenase dependent aggregation - ASPI test; vWf and glycoprotein Ib-dependent aggregation (using ristocetin) - RISTO test; Thrombin receptor activating peptide 6 (TRAP6)-induced activation of thrombin receptor (TRAP test), which is sensitive to Ⅱb/Ⅲa receptor antagonists

All of the platelet-activity tests were performed 30-180 min after blood collection, as suggested by the manufacturer.

Statistical analysis

The Shapiro-Wilk test was used to evaluate the distribution. Because of the non-Gaussian distribution, non-parametric tests were used. Differences in studied variables between groups were tested using the Mann-Whitney *U*-test and ANOVA range Kruskal-Wallis tests for independent groups. The correlations were analyzed with the Spearman rank correlation coefficient of $P < 0.05$ was considered statistically significant. The statistical analysis was performed with STATISTICA 7.0 (StatSoft Polska Sp z o.o., Krakow, Poland). The statistical review of the study was performed by a biomedical statistician.

RESULTS

Comparison of cirrhotic patients with and without PVT

A detailed comparison of analyzed groups regarding anthropometric, demographical and basic laboratory parameters is shown in Table 1.

When compared cirrhotic patients with and without PVT, there were significantly increased prothrombin levels in patients with PVT ($P = 0.01$). The TRAP test results were significantly lower in the PVT group ($P = 0.03$). No other differences in coagulation parameters or other platelet activity tests were found the groups. The comparison of analyzed coagulation factors and the results of the platelet activity tests are shown in Table 2.

Omentin serum concentrations were significantly decreased in patients with PVT ($P = 0.01$). There were no significant differences in vaspin levels between the groups. No significant differences were found in terms

Table 2 Coagulation parameters and platelet activity tests in cirrhotic patients with and without portal vein thrombosis

	PVT (+) *n* = 17	PVT (-) *n* = 34	*P* value
PT (s)	15.5 (12.5-21.9)	16.8 (11.3-44)	$P > 0.05$
Prothrombin activity (%)	67.8 (41-92)	66.5 (36-106)	$P > 0.05$
INR	1.37 (1.11-1.9)	1.36 (1-2.19)	$P > 0.05$
APTT	35.5 (29.1-57)	34.4 (23.6-49.4)	$P > 0.05$
TRAP test	37.2 (12-113)	55.6 (16-156)	$P = 0.03$
ASPI test	29.1 (5-108)	40.5 (5-130)	$P > 0.05$
ADP test	25.8 (1-94)	34.5 (5-99.9)	$P > 0.05$
RISTO low test	9.5 (1-35)	11.6 (0-33)	$P > 0.05$
RISTO high test	46.4 (5-162)	61.5 (4-156)	$P > 0.05$
D-dimer (mg/L)	4434 (663-9455)	3134 (563-11026)	$P > 0.05$
Protein C (%)	56.9 (38.1-90.3)	65.1 (20.9-177.5)	$P > 0.05$
Prothrombin (μg/mL)	175.6 (71-390)	117.4 (36-422)	$P = 0.01$
von Willebrand factor (mU/mL)	1872 (1328-2339)	1906 (981-2372)	$P > 0.05$
von Willebrand factor activity (IU/mL)	3.75 (1.72-6.61)	4.01 (1.01-7.71)	$P > 0.05$

P value < 0.05 was considered statistically significant. The data are shown as the mean and range or percentage where applicable. PT: Prothrombin time; INR: International normalized ratio.

Table 3 Adipokine serum levels, insulin resistance profile and diabetes occurrence in cirrhotic patients with and without portal vein thrombosis

	PVT (+) *n* = 17	PVT (-) *n* = 34	*P* value
Diabetes, *n* (%)	5/17 (29%)	10/34 (29%)	$P > 0.05$
Glucose (mg/dL) (N:75-115 mg/dL)	128.4 (86-309)	122.8 (82-430)	$P > 0.05$
Insulin (μIU/mL)	19.5 (8.7-41.7)	17.8 (6.1-47.6)	$P > 0.05$
HOMA-IR	6.6 (2.5-14.1)	5.3 (0.9-13.4)	$P > 0.05$
Omentin (ng/mL)	855 (579-1205)	1105 (619-2208)	$P = 0.01$
Vaspin (ng/mL)	0.31 (0.09-0.84)	0.27 (0.09-0.78)	$P > 0.05$

P values < 0.05 were considered statistically significant.

of diabetes mellitus occurrence, fasting glucose, fasting insulin levels, or HOMA-IR. The details are described in Table 3.

Results according to Child-Pugh score

Data comparing patients with various Child-Pugh (C-P) scores are presented in Table 4.

We observed that the APTT was significantly longer in C-P B patients than in the C-P A group. Moreover, D-dimer levels were increased slightly with more severe cases of liver disease. There was a significant decrease in prothrombin activity in more advanced liver disease cases. Von Willebrand factor levels and activity were significantly higher in C-P C group compared to the C-P A group.

No significant differences in platelet activity or adipokines serum levels were found with respect to liver disease severity as assessed with the C-P score.

Results according to MELD score

The patients were also divided into two groups based on MELD score (MELD $\leqslant$ 18 and > 18). A significantly shorter prothrombin time, activity and INR were confirmed in patients with MELD scores > 18. In contrast to the Child-Pugh score, there was a significant increase in the results of the TRAP-, ASPI- and RISTO tests in patients with lower MELD scores. The TRAP test values were higher in patients with less severe liver disease (56.2 *vs* 42.1, $P = 0.03$). The ASPI test (45.9 *vs* 26.4, $P = 0.001$) and RISTO-high test results were also higher in this group (68.5 *vs* 43, $P = 0.02$). On the other hand, von Willebrand factor levels were significantly higher in the MELD > 18 group (Table 5).

There were no significant differences with respect to omentin levels, vaspin levels, or metabolic parameters in the groups of patients with different MELD score.

Comparison of cirrhotic patients with viral vs non-viral and alcoholic vs non-alcoholic etiology

No significant differences were found between patients with viral and non-viral cirrhosis with respect to the analyzed adipokines and metabolic and coagulation factors (Table 6).

When compared to patients with alcoholic and non-alcoholic cirrhosis, prothrombin levels appeared to be higher in the latter. There was no difference in terms of omentin, vaspin, metabolic factors or other coagulation parameters between these two groups (Table 7).

Comparison of adipokine concentrations in cirrhotic patients with and without diabetes and different HOMA-IR levels

There was no significant difference in serum omentin or vaspin levels between cirrhotic patients with and without diabetes (991.0 ± 352.7 ng/mL *vs* 1035 ± 330.6 ng/mL, 0.20 ± 0.10 ng/mL *vs* 0.32 ± 0.23 ng/mL, respectively).

When compared cirrhotic patients with HOMA-IR > 3 *vs* $\geqslant$ 3, both serum omentin and vaspin levels were significantly down-regulated in patients with better insulin sensitivity (858.2 ± 196.0 ng/mL *vs* 1100.0 ± 355.1 ng/mL, $P = 0.03$; 0.17 ± 0.09 ng/mL *vs* 0.32 ± 0.19 ng/mL, $P = 0.02$, respectively).

Correlations between coagulation factors, platelet activity tests, metabolic factors, adipokines and clinical outcomes

A positive relationship between serum omentin and vaspin levels were found both when all of the patients were analyzed ($r = 0.41$, $P = 0.01$) and among those with PVT ($r = 0.94$, $P < 0.001$). In patients without PVT, the results were on the threshold of statistical significance ($P = 0.05$).

We found a positive correlation between insulin and omentin levels in PVT (+) patients ($r = 0.47$, $P = 0.04$). There was significant negative correlation between the presence of diabetes and the results of the platelet activity tests: ADP ($r = [-0.29]$, $P = 0.04$) and RISTO-low ($r = [-0.32]$, $P = 0.02$).

Table 4 Coagulation factors and platelet activity test results with regards to the severity of cirrhosis, as assessed according to the Child-Pugh score

	All patients (n = 51)	Child-Pugh A (n = 16)	Child-Pugh B (n = 25)	Child-Pugh C (n = 10)
Thrombosis	17/51 (33%)	6/16 (37.5%)	9/25 (24%)	2/10 (20%)
PT (s)	13.8 (11.3-44)	13.8 (11.3-16.9)	16.7 (11.3-44)[1,3]	19.2 (14.1-24.4)[1,2]
Prothrombin activity (%)	67 (36-106)	79.2 (58-106)	65.4 (43-95)[1,3]	51.4 (36-73)[1,2]
INR	1.3 (1-2.2)	1.2 (1-1.5)	1.4 (1.1-2)[1,3]	1.7 (1.2-2.2)[1,2]
APTT	34.7 (23.6-57)	32.3 (23.6-47.4)	36.2 (29.1-57)[1]	35.6 (28.2-44)
TRAP test	49.6 (12-156)	54.5 (15-156)	43.5 (12-98)	53.3 (16-113)
ASPI test	36.8 (5-130)	45.6 (10-130)	32.3 (5-110)	34.2 (5-108)
ADP test	31.6 (1-99.9)	36.3 (13-99.9)	25.8 (1-77)	38.4 (9-94)
RISTO low test	10.9 (0-35)	10.5 (1-25)	8.9 (0-26)	16.4 (2-35)
RISTO high test	56.5 (4-162)	65.5 (7-152)	41 (4-156)	60.49 (17-162)
D-dimer (mg/L)	3568 (563-11026)	2521 (563-9455)	3243 (795-8790)[3]	6053 (724-11026)[1,2]
Protein C (%)	56.5 (20.9-177.5)	60.9 (49.1-105.1)	60.8 (20.9-177.5)	58.3 (29.4-121.1)
Prothrombin (μg/mL)	136.8 (36-422)	128.9 (43.1-390)	152.1 (36-422)	110.2 (60.4-194)
vWf (mU/mL)	1941 (981-2372)	1789 (986-2372)	1941 (981-2339)	2062 (1450-2311)[1]
vWf activity (IU/mL)	3.8 (1-7.7)	3.3 (1.5-7.6)	3.8 (1-7.7)	4.8 (2.6-6.6)[1]
Omentin (ng/mL)	1023 (579-2208)	942 (635-1432)	964 (579-2208)	1102 (657-1571)
Vaspin (ng/mL)	0.29 (0.1-0.8)	0.2 (0.1-0.6)	0.3 (0.1-0.8)	0.4 (0.1-0.7)

[1]Significantly different *vs* Child-Pugh A group; [2]Significantly different *vs* Child-Pugh B group; [3]Significantly different *vs* Child-Pugh C group. P value < 0.05 was considered statistically significant. The data are shown as the mean and range or percentage where applicable. PT: Prothrombin time; INR: International normalized ratio; vWf: von Willebrand factor.

Table 5 Coagulation factors and platelet activity test with regards to the severity of cirrhosis, as evaluated according to the model of end-stage liver disease score

	All patients (n = 51)	MELD ⩽ 18 (n = 28)	MELD > 18 (n = 23)
Thrombosis	17/51 (33%)	11/28 (39.2%)	7/23 (30.4%)
PT (s)	13.8 (11.3-44)	14.1 (11.3-21)	19.1 (13.4.-44)[1]
Prothrombin activity (%)	67 (36-106)	76.4 (43-106)	55.6 (36-86)[1]
INR	1.3 (1-2.2)	1.3 (1-1.5)	1.6 (1.2-2.2)[1]
APTT	34.7 (23.6-57)	35.1 (23.6-57)	34.4 (28.2-44.9)
TRAP test	49.6 (12-156)	56.2 (15-156)	42.1 (12-113)[1]
ASPI test	36.8 (5-130)	45.9 (10-130)	26.4 (5-108)[1]
ADP test	31.6 (1-99.9)	36.2 (7-99.9)	26.5 (1-94)
RISTO low test	10.9 (0-35)	10.6 (1-25)	11.3 (0-35)
RISTO high test	56.5 (4-162)	68.5 (7-156)	43 (4-162)[1]
D-dimer (mg/L)	3568 (563-11026)	3094 (563-9455)	4144 (724-11026)
Protein C (%)	56.5 (20.9-177.5)	58.7 (20.9-105.1)	66.7 (29.4-177.5)
Prothrombin (μg/mL)	136.8 (36-422)	143.6 (36-390)	128.5 (59-422)
vWf (mU/mL)	1941 (981-2372)	1841 (986-2372)	2006 (981-2311)[1]
vWf (IU/mL)	3.8 (1-7.7)	3.7 (1.5-7.6)	4.2 (1-7.7)
Omentin (ng/mL)	1023 (579-2208)	948 (579-1432)	1110 (619-2208)
Vaspin (ng/mL)	0.29 (0.1-0.8)	0.25 (0.1-0.8)	0.32 (0.1-0.8)

[1]P value < 0.05 was considered statistically significant. MELD ⩽ 18 *vs* MELD > 18. The data are shown as the mean and range or percentage where applicable. PT: Prothrombin time; INR: International normalized ratio; vWf: von Willebrand factor.

Vaspin serum levels were negatively associated with diabetes occurrence (r = [-0.64], P = 0.04) and protein C concentration (r = [-0.63], P = 0.04). Vaspin levels were positively associated with von Willebrand factor levels (r = 0.36, P = 0.02). Omentin serum concentrations were positively related to von Willebrand factor levels (r = 0.40, P = 0.008).

There was no relationship between the grade of esophageal varices and serum vaspin or omentin levels.

DISCUSSION

Adipokines, adipose tissue-derived hormones, have been shown to have a variety of local, peripheral and central effects[6]. A growing number of studies show a particularly important role of adipokines in the development of liver damage in a variety of diseases[6,7,27,28]. However, relatively little research has been done concerning the influence of adipokines on the natural history of liver cirrhosis. To the best of our knowledge, this is the first study to assess omentin and vaspin levels in cirrhotic patients with PVT, the diagnosis of which remains challenging. It has been demonstrated that PVT generally coexists with a more severe course of cirrhosis. It is therefore essential to identify patients who are at risk for this complication.

Table 6 Coagulation factors, platelet activity tests and adipokines levels with regards to the presence of chronic hepatitis C

	All patients (n = 51)	HCV (+) (n = 15)	HCV (-) (n = 36)
Thrombosis	17/51 (33%)	3/11 (27.3%)	14/40 (35%)
PT (s)	13.8 (11.3-44)	14.5 (11.3-19.9)	16.8 (11.3-44)
Prothrombin activity (%)	67 (36-106)	72.1 (47-94)	65.6 (36-106)
INR	1.3 (1-2.2)	1.3 (1-1.8)	1.4 (1.2-2.2)
APTT	34.7 (23.6-57)	36.6 (28.2-57)	34.8 (23.6-49.4)
TRAP test	49.6 (12-156)	53.3 (15-156)	47.5 (12-113)
ASPI test	36.8 (5-130)	37.9 (10-130)	36.5 (5-110)
ADP test	31.6 (1-99.9)	33.4 (13-99.9)	31.2 (1-94)
RISTO low test	10.9 (0-35)	13.5 (4-25)	10.2 (0-35)
RISTO high test	56.5 (4-162)	44.5 (7-115)	60.1 (4-162)
D-dimer (mg/L)	3568 (563-11026)	3317 (595-10043)	3637 (563-11026)
Protein C (%)	56.5 (20.9-177.5)	65.1 (41.4-105.1)	61.6 (20.9-177.5)
Prothrombin (μg/mL)	136.8 (36-422)	149.2 (59.7-390)	133.4 (36-422)
vWf (mU/mL)	1941 (981-2372)	1912 (986-2311)	1890 (981-2372)
vWf activity (IU/mL)	3.8 (1-7.7)	4.2 (1.5-6.4)	3.9 (1-7.7)
Omentin (ng/mL)	1023 (579-2208)	991 (731-1432)	1028 (579-2208)
Vaspin (ng/mL)	0.29 (0.1-0.84)	0.43 (0.1-0.77)	0.26 (0.1-0.84)

[1]P value < 0.05 was considered statistically significant. The data are shown as the mean and range or percentage where applicable. PT: Prothrombin time; INR: International normalized ratio; vWf: von Willebrand factor; HCV: Hepatitis C virus.

Table 7 Coagulation factors, platelet activity tests and adipokines levels with regards to the etiology of alcoholic liver cirrhosis

	All patients (n = 51)	Alcoholic cirrhosis (n = 30)	Non-alcoholic cirrhosis (n = 21)
Thrombosis	17/51 (33%)	9/30 (30%)	8/21 (38%)
PT (s)	13.8 (11.3-44)	17 (11.3-44)	15.3 (11.3-21)
Prothrombin activity (%)	67 (36-106)	65.7 (36-106)	68.8 (43-94)
INR	1.3 (1-2.2)	1.5 (1-2.2)	1.4 (1.1-2)
APTT	34.7 (23.6-57)	34.6 (23.6-47.4)	34.8 (28.2-57)
TRAP test	49.6 (12-156)	51.2 (16-113)	47.2 (12-156)
ASPI test	36.8 (5-130)	39.1 (5-110)	33.4 (5-130)
ADP test	31.6 (1-99.9)	32.8 (5-94)	29.9 (1-99.9)
RISTO low test	10.9 (0-35)	11.4 (0-35)	10.15 (1-25)
RISTO high test	56.5 (4-162)	68.4 (10-162)	39.5 (4-115)
D-dimer (mg/L)	3568 (563-11026)	3635 (563-11026)	3471 (595-10043)
Protein C (%)	56.5 (20.9-177.5)	59.2 (20.9-121.2)	66.8 (33.4-177.5)
Prothrombin (μg/mL)	136.8 (36-422)	116.6 (36-382)	165.8 (59-422)[1]
vWf (mU/mL)	1941 (981-2372)	1937 (1209-2372)	1834 (981-2311)
vWf (IU/mL)	3.8 (1-7.7)	4.1 (1.5-7.7)	3.7 (1-6.4)
Omentin (ng/mL)	1023 (579-2208)	1072 (579-2208)	923 (635-1432)
Vaspin (ng/mL)	0.29 (0.1-0.84)	0.29 (0.1-0.84)	0.26 (0.1-0.72)

[1]P value < 0.05 was considered statistically significant. The data are shown as the mean and range or percentage where applicable. PT: Prothrombin time; INR: International normalized ratio; vWf: von Willebrand factor.

Our study for the first time showed significantly lower levels of serum omentin in cirrhotic patients with PVT. However, omentin serum levels were not associated with the severity of cirrhosis according to either the Child-Pugh or MELD scales. There was also no relationship between omentin and the grade of esophageal varices. Patients with PVT had significantly higher prothrombin concentrations. Prothrombin is a key factor in clot formation, and elevated levels appear to promote venous thrombosis. In the study by Eisinger *et al*[19], omentin levels were significantly higher in the portal vein. In addition, these levels tended to be higher in the hepatic vein and systemic blood of patients with liver cirrhosis compared with the respective blood compartments of control patients with healthy livers. Similar to our results, no association with complications resulting from portal hypertension was observed.

As mentioned above, there are conflicting results regarding omentin synthesis in the liver[14,15]. Our previous study of non-obese CHC patients, 16% of whom were cirrhotic, showed significantly higher omentin serum levels compared to healthy controls. However, omentin serum levels were not associated with any histopathological findings[14]. Similar results were found by Nassif *et al*[18]'s study, which showed significantly higher serum omentin levels in CHC subjects; however, no information was provided regarding histopathological examination.

In patients with obesity and metabolic syndrome, circulating omentin levels were negatively associated with a multiplicity of metabolic risk factors, suggesting

that omentin acts as a biomarker of metabolic disorders[16]. IR, which is considered to be negatively associated with serum omentin in patients with metabolic diseases, is commonly present in patients with liver cirrhosis. Therefore, higher serum omentin levels in cirrhotic patients with increased IR is an unexpected finding. However, our results are in accordance with results obtained by Eisinger *et al*[19], who found omentin serum levels to be significantly decreased in cirrhotic patients with better insulin sensitivity.

Nassif *et al*[18] analyzed CHC patients and found a negative correlation between serum omentin and HOMA-IR and fasting glucose, with lower serum omentin levels in subjects with T2DM. However, in those patients, serum omentin concentration was still significantly higher than in diabetic patients without CHC. We must still bear in mind the strong influence of DM and obesity on serum omentin levels[29,30]. An additional factor is the direct impact of HCV on inflammatory, metabolic and intracellular insulin pathways[31]. In the present study, there was no difference in serum omentin levels in cirrhotic patients with and without DM. This is an unexpected result considering the negative correlation between serum omentin and metabolic abnormalities in DM and MS. However, the result must be analyzed with caution due to a small number of patients with DM in our group. Nevertheless, in the study by Yilmaz *et al*[32], serum levels of omentin were significantly lower in normal controls compared to non-cirrhotic patients with biopsy-proven NAFLD, 40% of whom were diabetic and over 60% of whom had metabolic syndrome. Moreover, serum omentin appeared to be a predictor of hepatocyte ballooning, independent of potential confounders, including metabolic parameters. This last observation points to a pivotal role omentin in the development and progression of NAFLD.

To exclude a potential influence of HCV on IR and lipid profile, which may interfere with omentin serum levels, we compared cirrhotic patients with CHC to the rest of the study group. There were no significant differences between these two subgroups in terms of serum omentin, glucose and fasting insulin, HOMA-IR, cholesterol, coagulation factors or platelet activity.

The top three causes of cirrhosis in our study were alcoholic liver disease, CHC and autoimmune hepatitis. There were no differences in serum omentin levels in patients with viral or toxic cirrhosis compared to the rest of analyzed group. In the study by Eisinger *et al*[19], only three patients out of 34 had cirrhosis due to CHC. The comparison between our study and Nassiff *et al*[18]*'s* study is also difficult because no information was provided regarding the viral genotype and fibrosis stage in the analyzed patients. Therefore, the discrepancy in the obtained results regarding the relationship between serum omentin and insulin sensitivity may result from different study group characteristics.

In terms of to higher serum omentin levels in patients with CLDs, the question arises of whether these increased levels result from the impaired metabolism of this adipokine in the inflamed and fibrotic liver. However, our study did not show any difference in serum omentin concentrations in more advanced cirrhosis (*i.e*., more impaired liver function), suggesting no significant influence of hepatic metabolism on the levels of this adipokine. Therefore, further questions remain as to whether omentin expression in the liver is an important source of the serum levels of this adipokine. Our previous study confirmed omentin liver expression. However, hepatic gene expression was not associated with its serum levels or any histopathological feature. Moreover, omentin was not up-regulated in cirrhosis[14].

The dysfunction of SECs in cirrhotic liver is strictly associated with a low production of vasodilators, such as nitric oxide, which increases intrahepatic resistance and portal hypertension[33,34]. Nitric oxide is a central molecule in the regulation of vascular tone by regulating eNOS activity in blood vessels[35]. Omentin was found to mediate endothelium relaxation by up-regulating eNOS activity[12]. Omentin has also been described as a potent anti-inflammatory adipokine, inhibiting TNF alpha-mediated phosphorylation of p38 kinase and Jun kinase in vascular smooth muscle cells[36]. Therefore, higher omentin levels in patients with cirrhosis may be a compensatory mechanism against intrahepatic resistance.

Vaspin is another novel adipokine that is primarily produced by visceral and subcutaneous adipose tissue. The current study for the first time compared serum vaspin levels in cirrhotic patients with and without PVT and did not find any difference between these groups. Our previous study in CHC patients suggested serum vaspin to be a potential predictor of advanced liver fibrosis, with evident increases in subjects with advanced fibrosis[21]. However, in patients with insignificant fibrosis, serum vaspin level were significantly lower than in controls. Similarly, serum vaspin levels were lower in NAFLD patients with simple steatosis compared to healthy controls, with a subsequent increase in patients with NASH and ballooning degeneration[23]. The study by Aktas *et al*[24] confirmed serum vaspin to be a predictor of liver fibrosis in NAFLD, independently of potential confounders, including metabolic parameters. Moreover, vaspin serum levels reflected the intensity of hepatic angiogenesis in CHC patients, a phenomenon that aggravates CLDs progression[22]. It is well established that active fibrogenesis and angiogenesis are connected with the progression of portal hypertension, an undisputed risk factor of PVT. In the present study, there was no difference in vaspin serum levels between patients with different stages of cirrhosis according to Child-Pugh and MELD scores. This is in accordance with previous results showing that there is no further increase in vaspin serum levels when advanced fibrosis

appears[21,22].

Visceral vaspin expression significantly correlated with BMI, percentage of body fat and the levels of plasma glucose. As vaspin has been found to be a compensatory adipokine in IR, a common metabolic disorder in patients with cirrhosis, serum vaspin is expected to be higher in patients with worsen insulin sensitivity. As expected, the present study revealed significantly higher serum vaspin levels in patients with HOMA-IR $\geqslant$ 3. The results with respect to vaspin levels are in accordance with our previous study in NAFLD patients, which showed HOMA-IR to be significantly higher in patients with fibrosis and to correlate with fibrosis stage[23]. In contrast, serum vaspin levels had no tendency to be increased in CHC patients with HOMA-IR $\geqslant$ 3[37]. Bearing in mind a direct influence of HCV on insulin sensitivity, further analysis comparing serum vaspin levels in groups of patients with viral and non-viral cirrhosis was carried out. However, no significant differences were observed between these two subgroups of cirrhotic patients. Similarly, no difference was detected when comparing patients with toxic and non-toxic cirrhosis. These observation support the opinion that increased serum vaspin levels may be a compensatory mechanism to abolish IR.

Recently, vaspin has been found to exert anti-atherogenic actions. Vaspin decreased the levels of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of all types of nitric oxide synthases (NOSs), and activated dimethylarginine dimethylaminohydrolase (DDAH), an enzyme that metabolizes ADMA to citrulline and dimethylamine. Elevated ADMA has been shown to attenuate endothelium-dependent vasodilation in humans[38] and, together with dysregulation of DDAH, is involved in endothelial dysfunction in hypercholesterolemia and diabetes. Some recent evidence has indicated that DDAH activity is impaired by oxidative stress, permitting ADMA to accumulate[39]. Considering the beneficial effect of vaspin on IR and oxidative stress, it may act as a compensatory and protective factor. Unfortunately, our study did not reveal any difference in vaspin serum levels between cirrhotic patients with and without PVT, and there was no relationship with the grade of esophageal varices. The evident limitation of our study is the lack of portal pressure measurement; therefore, the interpretation of the presented results should be performed with caution.

As mentioned above, PVT is more frequently observed in later stages of cirrhosis. However, the development of PVT is unpredictable, and its risk factors are not well characterized. According to Virchow's triad principle, a venous thrombosis results from the coexistence of blood flow abnormalities, endothelial injury and hypercoagulation. For these reasons, PVT in cirrhosis could be linked with endotoxemia, thrombophilia and portal hypertension. Alternatively, PVT it may have no definite association with any of these factors. Advanced cirrhosis is associated with profound and complex coagulation defects, with concomitant defective fibrinolysis and impaired synthesis of thromboxane A2 and serotonin by platelets. The final result of all of these disturbances is a promotion of a prothrombotic state, likely related to up-regulated endothelial synthesis of von Willebrand factor (vWf) and increased levels of Ⅷ factor. These effects occur in combination with low levels of hepatic anticoagulation agents, such as antithrombin Ⅲ and proteins C and S[40,41]. vWf levels and the Ⅷ factor-to-PC ratio are as predictive of mortality as MELD. Thrombogenic mechanisms that operate within the cirrhotic liver might underlie the progression of portal hypertension and adverse clinical outcomes in patients with cirrhosis[42].

Our study revealed prothrombin concentration to be significantly higher in patients with PVT. Prothrombin is an essential agent that participates in clot formation. Elevated levels of this factor lead to thrombin formation, inhibit the activity of antithrombotic protein S, and promote venous thrombosis in the presence of endothelial injury[43,44]. On the other hand, in our study, protein C levels tended to be lower in PVT patients, although the difference did not reach statistical significance.

The loss of platelet inhibition by insulin has been suggested to be a major determinant of platelet hyperactivity during obesity. Low levels of HDL-cholesterol promote platelet activation and aggregation, possibly because HDL antagonizes the stimulating properties of LDL on platelets[45]. In agreement with these facts, we found that not HDL-cholesterol but also LDL-cholesterol and total blood cholesterol levels were markedly diminished in patients with PVT; however, the difference was not statistically significant. The roles of omentin and vaspin in decreasing IR and oxidative stress may exert a protective effect against platelet hyperreactivity. Unfortunately, serum omentin was not associated with platelet hyperactivity. On the other hand, both serum omentin and vaspin concentrations, in addition to fasting insulin, were positively related to vWF level and activity.

However, the tendency of patients with liver disease to bleed and to experience thrombus formation cannot be explained solely by alterations in hemostasis. A partial explanation of hemorrhagic and thrombotic events in patients with advanced CLDs or cirrhosis result from complex hemodynamic alterations, such as portal hypertension, endothelial dysfunction, kidney dysfunction, and the release of substances similar to heparin by bacterial[46].

The last intriguing observation of the study, which has not been previously described, is a positive association between serum omentin and vaspin. As mentioned above, ADMA, an endogenous inhibitor of NOS, is increased in the serum of patients with liver cirrhosis compared with healthy controls and may antagonize peripheral vasodilatation. High intrahepatic ADMA levels may aggravate intrahepatic resistance[47,48]. The ability of vaspin and omentin to enhance eNOS

activity and to down-regulate ADMA production may be a compensatory and protective mechanism against portal hypertension. The positive mutual correlation between both adipokines may suggest a collaborative action of both adipokines, not only as compensatory agents in portal hypertension but also in insulin resistance, oxidative stress and inflammation.

There are some evident limitations of our study. First, the group of patients is relatively small and not homogenous. Second, no liver biopsy was carried out, and the measurement of collagen proportionate area, which may reflect the severity of cirrhosis and predict portal hypertension, was not applicable. Third, no measurement of portal pressure was made, so a direct assessment of how omentin and vaspin may influence vasodilatation or vasoconstriction was not possible. Fourth, the relationship between PVT and the levels of omentin is a simple association and not a cause-effect relationship. A follow-up study should have been designed to demonstrate that decreased omentin levels are related to a higher risk of developing PVT.

In conclusion, our study showed serum omentin but not vaspin levels to be significantly higher in cirrhotic patients without PVT. Moreover, omentin and vaspin concentrations were higher in cirrhotic patients with increased IR. Neither omentin nor vaspin were associated with other metabolic abnormalities, including DM. The origin of liver cirrhosis and grade of liver impairment as assessed by the Child-Pugh or MELD scores were not correlated with omentin or vaspin levels. There was no relationship between platelet hyperactivity and the serum levels of either analyzed adipokine. The positive mutual correlation between omentin and vaspin may suggest their collaborative action against IR, inflammation and portal hypertension. Our results indicate that although omentin and vaspin could be involved into the pathophysiology of the development of cirrhosis, they are not good indicators of its origin or severity and do not impact the thrombotic activity of platelets.

Additional studies must delineate whether the levels of omentin and vaspin play a pivotal, protective role in liver cirrhosis against PVT and portal hypertension.

COMMENTS

Background

Ongoing research has revealed alterations in the levels and expression of various adipokines in cirrhosis. Portal vein thrombosis (PVT) has been considered to be a complication of more advanced liver cirrhosis. The data regarding novel adipokines in liver cirrhosis is scare and ambiguous. The current study evaluated the serum concentrations of omentin and vaspin in patients with liver cirrhosis of different origins and stages with and without PVT. The relationships of these measures with disease severity and etiology, platelet activity, hemostatic parameters and potential complications were also assessed.

Research frontiers

Accumulating data suggest that obesity and insulin resistance are related to a more rapid progression of chronic liver diseases, the development of cirrhosis and its complications. Some adipokines have been suggested to contribute to the complicated pathophysiology of hepatic injury and repair. Moreover, there is some data suggesting, that omentin plays role in regulating endothelial homeostasis.

Innovations and breakthroughs

This study showed serum omentin levels to be significantly higher in cirrhotic patients without PVT. This finding confirms important role of this adipokine in vessel homeostasis. Serum levels of omentin and vaspin seem not to be associated with platelet hyperactivity.

Applications

The presented study suggests that there is a need of further studies on the role of novel adipokines in liver cirrhosis and its complications as liver failure is strictly connected to metabolism disturbance.

Peer-review

Interesting and certainly new topic describing two novel adipokines - omentin and vaspin in patients with liver cirrhosis with and without PVT. The study provides analysis of relationship between adipokines serum levels and disease severity and etiology, metabolic abnormalities and platelets activity.

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ORIGINAL ARTICLE

Observational Study

Upper gastrointestinal cancer burden in Hebei Province, China: A population-based study

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Institutional review board statement: All procedures involving human participants were performed in accordance with the ethical standards and with the 2013 Helsinki declaration and its later amendments. The study was reviewed and approved by the Cancer Institute, the Fourth Hospital of Hebei Medical University, Shijiazhuang, China

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Abstract

AIM

To investigate the incidence and mortality rates of upper gastrointestinal cancer (UGIC) in Hebei Province, China, and to identify high-risk populations to improve UGIC prevention and control.

METHODS

Data for UGIC patients were collected from 21 population-based cancer registries covering 15.25% of the population in Hebei Province. Mortality data were extracted from three national retrospective death surveys (1973-1975, 1990-1992 and 2004-2005). The data were stratified by 5-year age groups, gender and area (high-risk/non-high-risk areas) for analysis. The age-period-cohort and grey system model were used.

RESULTS

The crude incidence rate of UGIC was 55.47/100000, and the adjusted rate (Segi's population) was 44.90/100000. Males in rural areas had the highest incidence rate (world age-standardized rate = 87.89/100000). The

crude mortality rate of UGIC displayed a decreasing trend in Hebei Province from the 1970s to 2013, and the adjusted rate decreased by 43.81% from the 1970s (58.07/100000) to 2013 (32.63/100000). The mortality rate declined more significantly in the high-risk areas (57.26%) than in the non-high-risk areas (55.02%) from the 1970s to 2013. The median age at diagnosis of UGIC was 65.06 years in 2013. There was a notable delay in the median age at death from the 1970s (66.15 years) to 2013 (70.39 years), especially in the high-risk areas. In Cixian, the total trend of the cohort effect declined, and people aged 65-69 years were a population at relatively high risk for UGIC. We predicted that the crude mortality rates of UGIC in Cixian and Shexian would decrease to 98.80 and 133.99 per 100000 in 2018, respectively.

CONCLUSION

UGIC was the major cause of cancer death in Hebei Province, and males in rural areas were a high-risk population. We should strengthen early detection and treatment of UGIC in this population.

Key words: Upper gastrointestinal cancer; Incidence; Mortality; Age-period-cohort; Grey system model

Core tip: This study is the first to examine the incidence and mortality trends of UGIC in Hebei Province. Data were collected from 21 population-based cancer registries covering 15.25% of the total population of Hebei Province in 2013. An age-period-cohort model was established to analyse the incidence rate of UGIC in a high-risk area of Hebei Province (Cixian). Additionally, we established the grey system model to predict the mortality rates of UGIC in high-risk areas of Hebei Province (Cixian and Shexian). The aim of the study was to provide epidemiological evidence for developing strategies for UGIC prevention and control.

Li DJ, Liang D, Song GH, Li YW, Wen DG, Jin J, He YT. Upper gastrointestinal cancer burden in Hebei Province, China: A population-based study. *World J Gastroenterol* 2017; 23(14): 2625-2634 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i14/2625.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i14.2625

INTRODUCTION

According to GLOBOCAN 2012, upper gastrointestinal cancer (UGIC) is the third most common cancer worldwide and the second leading cause of death among all cancer types. Approximately 45% of all cases occur in China[1]. The incidence and mortality proportions of UGIC are approximately 19.8% and 23.3% of all malignancies in China, respectively[2]. Thus, monitoring and studying the incidence and mortality of UGIC can provide important information, enable effective assessment and potentially generate a strategy for UGIC prevention and control based on its distribution pattern.

Hebei Province is located in northern China along the Taihang Mountain chain and has a relatively less developed economy. This province was recognized as a high-risk area for UGIC in the 1970s. In 2015, Hebei Province had 21 population-based cancer registries that covered 11185626 registered individuals (approximately 15.25% of the total population of Hebei Province). Additionally, Hebei Province participated in three national death surveys that were conducted during the periods from 1973-1975[3], 1990-1992[4] and 2004-2005[5]. Cixian and Shexian in Hebei Province exhibit a high frequency of UGIC; their cancer registries were established in 1974 and 2000, respectively. Several investigations[6-8] have been conducted to assess the potential risk factors and the corresponding aetiological intervention methods and screening measures for UGIC.

This study combined the data for esophageal cancer and stomach cancer to investigate the real burden of UGIC in Hebei Province and to provide information on its prevention and treatment.

MATERIALS AND METHODS

Cancer registry data

The Hebei Provincial Cancer Registry was established in 2009 and is responsible for cancer data collection, evaluation and publication. Data were collected from 21 local population-based registries in 2013, including 16 counties (Cixian, Shexian, Qianxi County, Wuan County, Zanhuang County, Fengning County, Xinji County, Xingtai County, Zhangbei County, Anguo County, Haixing County, Yanshan County, Neiqiu County, Renxian, Xuanhua County and Shenze County) and 5 cities (Baoding City, Qinhuangdao City, Cangzhou City, Shijiazhuang City and Shuangqiao District of Chengde City). These counties and cities represented urban areas and rural areas, respectively. The reported cases were from multiple sources, including local hospitals, community health centres, the Urban Resident Basic Medical Insurance Program and the New Rural Cooperative Medical Scheme. All data on cancer in Hebei Province were obtained from the Hebei Provincial Cancer Registry Database. Population information was collected from the local statistical bureau or household register department in the local public security bureau. We also extracted data on UGIC in Cixian from 1989 to 2013 and Shexian from 2004 to 2013. According to the International Classification of Diseases, 10th revision (ICD-10), C15 (esophageal cancer) and C16 (gastric cancer), which are UGIC, were extracted for the analysis.

Quality control

The inclusion criteria for the data were based on the Guidelines of the Chinese Cancer Registration[9] and the International Agency for Research on Cancer/International Association of Cancer Registries (IARC/IACR). The analysed data had a morphological verification percentage (MV%) higher than 66%, a percentage of cancer cases identified with death certification only (DCO%) less than 15% and a mortality to incidence ratio (M/I) between 0.6 and 0.8. All of the data were checked and evaluated based on the data quality criteria.

National retrospective survey of mortality

In the mid-1970s, a nationwide retrospective survey of causes of mortality was conducted in 29 provinces, including all 153 cities and counties. This survey covered 47.725 million people in Hebei Province[3] and provided a profile of cancer mortality in Hebei Province.

The second national retrospective sampling survey of cancer mortality was conducted from 1990 to 1992. This survey used a stratified sampling method and covered approximately 10% of the population in China[4]. A total of 21 cities and counties, including 6.375 million people in Hebei Province, were enrolled in the sampling areas.

The third national retrospective stratified sampling survey of all causes of death was performed during the period from 2004-2005 in 31 provinces/municipalities/autonomous regions, which included Hebei Province. A total of 18 cities and counties were selected as sampling areas, covering 13.79 million people and 20.15% of the total population of Hebei Province[5].

The pooled data for UGIC from the three national retrospective sampling surveys were stratified by area (high-risk/non-high-risk areas) and gender. Cixian, Shexian and Zanhuang counties were selected as the high-risk areas; the rest counties were considered non-high-risk areas.

Statistical analysis

The data analyses were performed using SPSS version 19.0 (SPSS Inc., Chicago, IL, United States), SAS version 9.4 (SAS Institute Inc., Cary, NC, United States) and Microsoft Excel 2003 (Microsoft, United States). The world age-standardized rate (ASR World) and Chinese age-standardized rate (ASR China) were calculated based on Segi's population and the Chinese population in 2000, respectively. Long-term trends of UGIC in Cixian and Shexian were analysed using an age-period-cohort model and grey system (GM) (1, 1) model. We used an age-period-cohort model to estimate the relative risks by age, period, and birth cohort based on the Cixian data collected from 1989 to 2013. Additionally, we extracted thirteen 5-year age groups from 25-29 to 85+ years old and five 5-year periods ranging from 1989-1993 to 2009-2013 to produce birth cohorts from 1904-1908 to 1984-1988. The three components (*i.e.*, cohort, period, and age) in the age-period-cohort model were linearly dependent. Thus, we adopted the common method of including an additional arbitrary reference constraint for the period effect[10,11]. Additionally, the periods from 2004-2008 and 2009-2013 and the birth cohort from 1934-1938 were chosen as reference groups for the 85+ age group.

Based on the crude mortality rates of UGIC in Cixian and Shexian from 2004-2013, an Excel table was used to establish the GM (1, 1)[12]. The posterior error ratio (C) and small error probability (*P*) were used to determine the prediction accuracy, the relative error between the predicted value and the actual value, and the extrapolated prediction of mortality of UGIC in Cixian and Shexian from 2014-2018.

RESULTS

Incidence of UGIC in 2013

In 2013, there were 6205 new UGIC cases (4328 in males and 1877 in females), accounting for 24.62% of all types of cancer in the Hebei Provincial Cancer Registry areas. The crude incidence rate of the total areas was 55.47 per 100000 people. The ASR World and the ASR China in 2000 were 44.90 and 44.16/100000 people, respectively (Table 1). The incidence rate of UGIC was higher in males than in females. The incidence rate was 2.62 times higher in rural areas (75.17/100000) than in urban areas (28.69/100000). Almost 78% of the new cases were from rural cancer registries, and more than 50% of the cases were males from rural areas. The male incidence rate was highest in rural areas (101.35/100000). The median age at diagnosis of UGIC was 65.06 years (64.73 years in males and 66.03 years in females) in Hebei Province. The median age was 4.36 years younger in rural areas (64.37 years) than in urban areas (68.74 years).

The age-specific incidence rate was relatively low for individuals younger than 40 years of age and then increased dramatically. The incidence rate reached the peak in the 85+ age group (504.44/100000), although the incidence rate reached the peak in the 80-84 age group (598.17/100000) for males. The trend in the urban and rural areas was similar to the overall trend (Figure 1).

Age, period and cohort effects in Cixian

Generally, the trend of the incidence rates of UGIC decreased with the year of birth. The incidence rate increased after a decrease and then decreased again for individuals age older than 55 years but remained steady for the 25-to-54-year-old age groups (Figure 2).

Age, period and cohort effects contributed to the observed changes in the UGIC incidence. The model including all three components had the best

Table 1 Upper gastrointestinal cancer in Hebei Provincial Cancer Registry areas, 2013

Area	Gender	Upper gastrointestinal cancer				Esophageal cancer				Stomach cancer			
		No.	Crude rate ($1/10^5$)	ASR China ($1/10^5$)	ASR World ($1/10^5$)	No.	Crude rate ($1/10^5$)	ASR China ($1/10^5$)	ASR World ($1/10^5$)	No.	Crude rate ($1/10^5$)	ASR China ($1/10^5$)	ASR World ($1/10^5$)
Total	Both genders	6205	55.47	44.16	44.90	2349	21.00	16.63	16.91	3856	34.47	27.54	27.99
	Male	4328	75.8	62.43	63.50	1517	26.57	21.83	22.16	2811	49.23	40.60	41.34
	Female	1877	34.28	26.43	26.75	832	15.19	11.59	11.81	1045	19.08	14.84	14.94
Urban	Both genders	1360	28.69	21.73	21.65	449	9.47	7.12	7.16	911	19.22	14.61	14.49
	Male	969	40.46	32.51	32.5	315	13.15	10.51	10.48	654	27.31	22.00	22.02
	Female	391	16.67	12.03	11.74	134	5.71	4.00	4.05	257	10.96	8.03	7.69
Rural	Both genders	4845	75.17	62.3	63.75	1900	29.48	24.45	24.97	2945	45.69	37.85	38.79
	Male	3359	101.35	85.84	87.89	1202	36.27	30.92	31.67	2157	65.08	54.91	56.22
	Female	1486	47.46	38.79	39.69	698	22.29	18.05	18.37	788	25.17	20.73	21.31

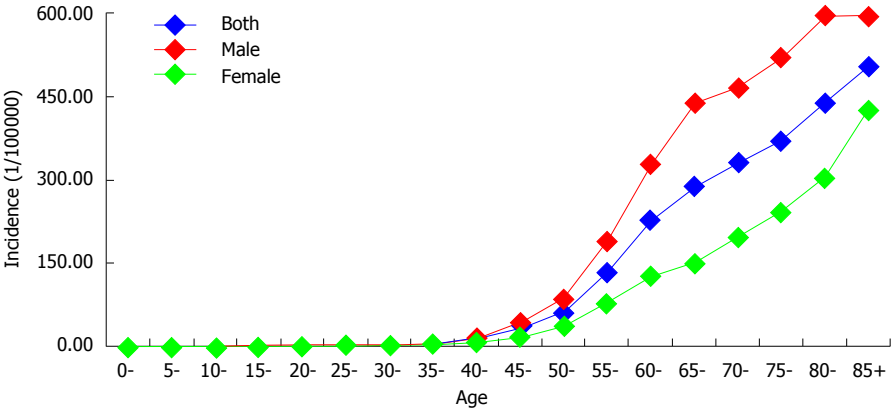

Figure 1 Age-specific incidence rates of upper gastrointestinal cancer in Hebei Province, 2013.

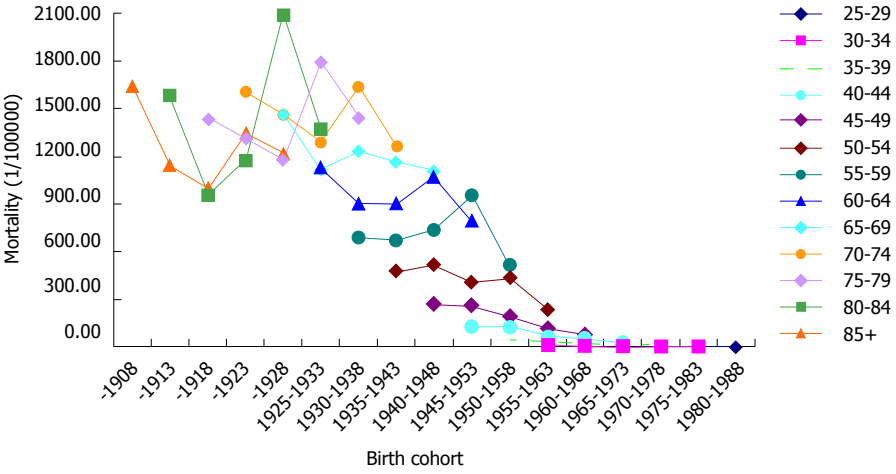

Figure 2 Trend in the age-specific incidence rate of upper gastrointestinal cancer plotted against the birth cohort.

goodness of fit, *i.e.*, the model had the lowest Akaike's information criterion value (691.267).

The incidence rate increased with age but decreased with the cohort. Figure 3 details the age effect and shows an increasing trend for individuals aged 25-65 years. The 65-69 age group had the highest relative risk (RR = 2.38) compared with the reference group (85+ age group). The birth cohort effect showed a consistent decline in the incidence rate in all cohorts after those born in the early 1900s. The RR of the period effects was lower from 1989-1993 compared to the reference group (Figure 3).

Mortality rates of UGIC in Hebei Province, 1970s-2013

A decreasing trend in the mortality rates of UGIC was observed in the period from 1973-1975 (50.53/100000) to 2013 (40.21/100000). The mortality rates increased slightly from the 1970s to 1990s

Table 2 Mortality rates of upper gastrointestinal cancer in Hebei Province from the 1970s to 2013

	1973-1975			1990-1992			2004-2005			2013		
	Both	Male	Female	Both	Male	Female	Both	Male	Female	Both	Male	Female
Deaths	72347	49516	22831	10429	7049	3380	7103	4927	2176	4498	3000	1498
Crude rate (1/10^5)	50.53	67.68	32.61	53.72	70.82	35.72	51.24	69.16	32.29	40.21	52.54	27.35
ASR China (1/10^5)	57.61	79.19	36.29	65.24	90.35	41.46	45.84	64.49	27.71	32.76	44.86	21.28
ASR World (1/10^5)	58.07	80.04	36.30	65.62	91.02	41.45	46.01	64.75	27.70	32.63	44.77	21.03
Ratio (%)	51.29	58.14	40.86	47.00	49.75	42.13	38.55	41.94	32.59	27.64	29.55	24.49
Median age (yr)	66.15	65.78	67.04	66.22	65.81	67.11	68.09	67.64	69.23	70.39	69.21	72.95

ASR China: Age-standardized rate based on China's standard population (2000); ASR World: Age-standardized rate based on the world standard population.

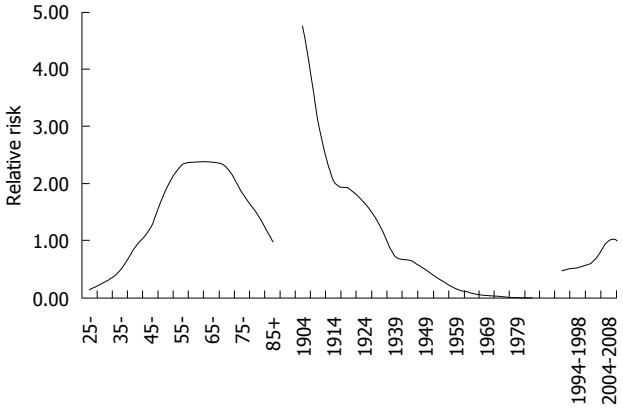

Figure 3 Estimation of age, period and birth cohort effects on the upper gastrointestinal cancer incidence in Cixian, 1989-2013.

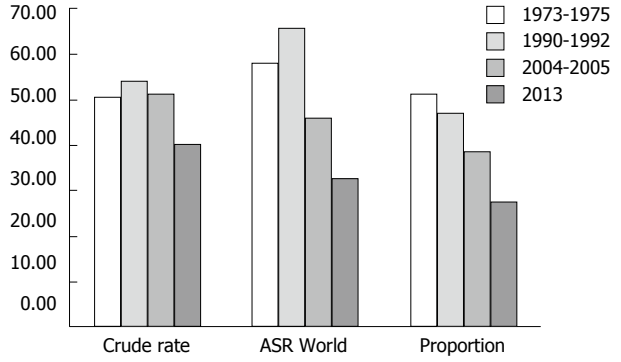

Figure 4 Mortality of upper gastrointestinal cancer in Hebei Province from the 1970s to 2013.

and then dropped sharply. In Hebei Province, the data from the four periods of the death survey indicated that the mortality rate in 2013 had decreased by 20.42%, 25.15% and 21.53% compared with the three periods from the national sampling surveys of causes of death (1973-1975, 1990-1992, and 2004-2005, respectively). Age standardization decreased the rates by 43.81%, 50.27% and 29.08%, respectively. For males, the ASR World in 1973-1975 (80.04/100000) was 1.79 times higher than that in 2013 (44.77/100000). In females, the rate decreased from 36.30/100000 in 1973-1975 to 21.03/100000 in 2013 (Table 2, Figure 4). The proportions of UGIC among all types of cancers were 51.29%, 47.00%, 38.55% and 27.64% during the periods from 1973-1975, 1990-1992, 2004-2005, and 2013, respectively, and showed a stepwise decrease, especially for males. The mortality rates of UGIC dropped in both the high-risk and non-high-risk areas from the 1970s to 2013. The mortality rates in 2013 decreased by 57.26%, 54.17% and 29.44% in the high-risk areas compared with the three periods from the sampling surveys of national causes of death (1973-1975, 1990-1992, and 2004-2005) and by 55.02%, 39.67% and 21.65% in the non-high-risk areas, respectively. The mortality rates in the high-risk areas were 3.76 times higher than those in the non-high-risk areas in 2013. Additionally, the ASR World ratio between the high-risk areas and non-high-risk areas was lower than those from the three death survey periods (3.95, 4.94 and 4.17, respectively) (Table 3).

According to the graph depicting the age-specific rates in different periods, the periods from 1973-1975, 2004-2005 and 2013 had similar trends. Beginning with the 45-49 age group, the mortality rate of UGIC increased. The mortality rate reached the peak in the 80-84 age group. In contrast, the mortality rate of the 70-74 age group was highest from 1990-1992. The age-specific mortality rates were lower in 2013 than in 1973-1975, 1990-1992 and 2004-2005 for those younger than 75 years of age but dramatically increased thereafter, with reports of mortality rates higher than the rates in the three sampling surveys of national causes of death (Figure 5).

Median age at death of UGIC patients in Hebei Province, 1970s-2013

The median age at death caused by UGIC gradually increased. The median was 66.15 years in 1973-1975 and 66.22 years in 1990-1992.The median age at death increased from 68.09 years in 2004-2005 to 70.39 years in 2013. The median age at death for females increased to 72.95 years in 2013.

There was a notable delay in the median age at death caused by UGIC from the 1970s to 2013 in the high-risk areas. The median age of death was delayed

Table 3 Mortality rates of upper gastrointestinal cancer in high-risk areas and non-high-risk areas of Hebei Province from the 1970s to 2013

	1973-1975				1990-1992				2004-2005				2013			
	Deaths	Crude rate ($1/10^5$)	ASR World ($1/10^5$)	Median age (yr)	Deaths	Crude rate ($1/10^5$)	ASR World ($1/10^5$)	Median age (yr)	Deaths	Crude rate ($1/10^5$)	ASR World ($1/10^5$)	Median age (yr)	Deaths	Crude rate ($1/10^5$)	ASR World ($1/10^5$)	Median age (yr)
High-risk areas																
Male	2975	214.94	286.44	62.99	3182	189.69	300.62	62.91	2005	158.63	188.08	65.46	969	143.19	131.41	69.22
Female	1536	118.93	150.76	64.70	1645	98.63	133.82	64.55	910	76.25	80.50	67.21	489	75.68	59.43	73.04
Both genders	4511	168.60	218.69	63.54	4827	140.79	203.94	63.48	2915	118.62	132.47	66.01	1458	110.22	93.46	70.37
Non-high-risk areas																
Male	46541	64.85	76.49	65.95	3867	46.73	58.14	66.10	2922	49.87	45.00	67.12	2031	40.36	34.22	69.21
Female	21295	30.99	34.39	67.22	1735	22.26	24.85	66.36	1266	22.83	18.91	68.11	1009	20.89	15.91	72.91
Both genders	67836	48.28	55.34	66.32	5602	35.04	41.26	66.18	4188	36.72	31.77	67.41	3040	30.82	24.89	70.40

ASR World: Age-standardized rate based on the world standard population.

Table 4 Mortality data analysis of upper gastrointestinal cancer *via* the grey system model in Cixian and Shexian

Year	t	Cixian			Shexian		
		Mortality rate	Predicted rate	Error	Mortality rate	Predicted rate	Error
2004	1	113.41			156.08		
2005	2	117.74	124.88	-7.13	158.66	160.24	-1.57
2006	3	126.51	129.78	-3.27	150.89	159.62	-8.73
2007	4	123.38	123.73	-0.35	145.89	164.62	-18.73
2008	5	122.51	118.66	3.85	167.40	172.49	-5.09
2009	6	123.59	112.34	11.25	168.63	156.75	11.88
2010	7	99.45	102.87	-3.42	148.73	137.70	11.02
2011	8	114.65	115.50	-0.85	147.39	136.52	10.88
2012	9	112.17	110.93	1.24	138.28	134.65	3.63
2013	10	106.87	106.87	0.00	139.88	139.90	-0.02
2014	11		106.18			141.57	
2015	12		104.29			139.64	
2016	13		102.42			137.73	
2017	14		100.59			135.85	
2018	15		98.80			133.99	

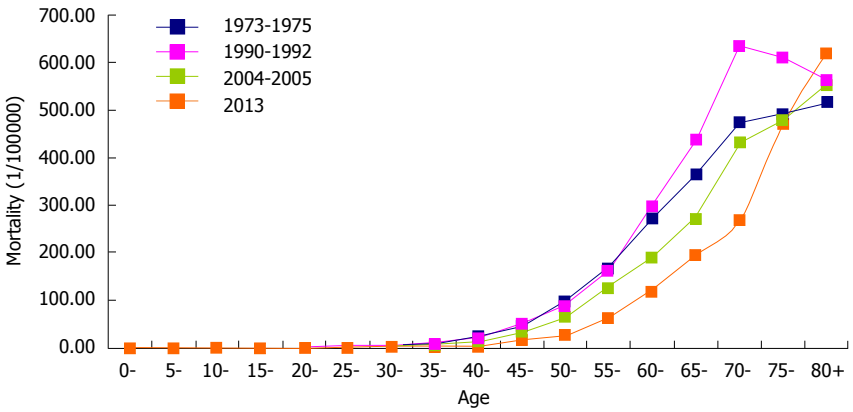

Figure 5 Age-specific mortality rates of upper gastrointestinal cancer in Hebei Province from the 1970s to 2013.

by 6.84 years in the high-risk areas compared to only 4.08 years in the non-high-risk regions. The median age at death for females was delayed by 8.34 and 5.69 years in the high-risk areas and non-high-risk areas, respectively. In 2013, the median age at death in the high-risk areas (70.37) was similar to that in the non-high-risk areas (70.40).

Mortality rate prediction for UGIC in Cixian

The mortality rate of UGIC in Cixian was 113.41 per 100000 in 2004. The mortality rate decreased to 106.87 per 100000 in 2013. The GM (1, 1) equation was $Y_t = -6992.90e^{-0.01802(t-1)} + 7106.31$, which predicted that the mortality rate of UGIC in Cixian would decrease to 98.80 per 100000 in 2018, with a

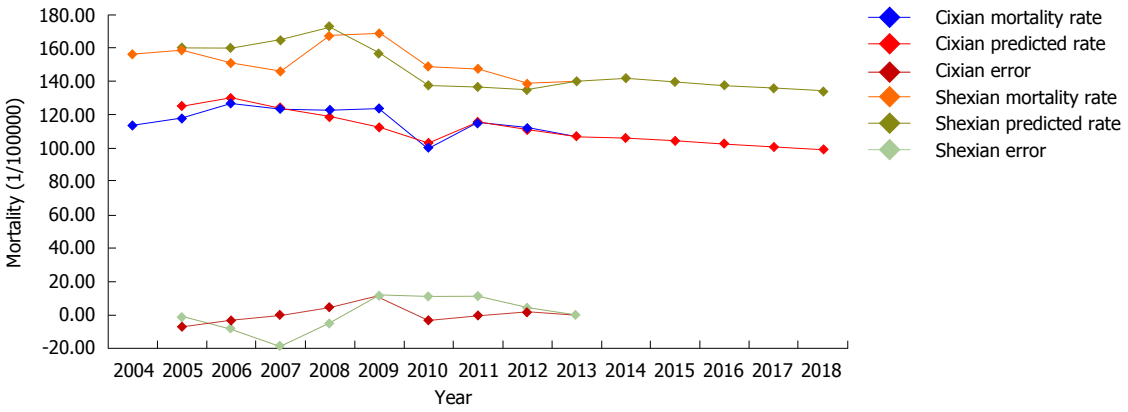

Figure 6 Mortality rate prediction of upper gastrointestinal cancer *via* the grey system model in Cixian and Shexian.

maximum relative error of 9.10%. The C was 0.58, and the *P* was 0.78, which indicated that the model was qualified (Table 4, Figure 6).

Mortality rate prediction for UGIC in Shexian

The mortality rate of UGIC in Shexian was decreased by 10.38% from 2004 to 2013. The GM (1, 1) equation was $Y_t = -11725.81e^{-0.01376(t-1)} + 11881.89$, which predicted that the mortality rate of UGIC in Shexian would decrease to 133.99 per 100000 in 2018, with a maximum relative error of 12.84%. The C and *P* were 0.94 and 0.44, respectively, indicating that the model was up to standard (Table 4, Figure 6).

DISCUSSION

This study is the first to examine the incidence and mortality trends of UGIC in Hebei Province, China. The study collected data from 21 population-based cancer registries covering 15.25% of the total population of Hebei Province in 2013, which had not achieved maximal coverage in this province to date. The aim was to provide epidemiological evidence for developing strategies to control UGIC. The crude incidence rate of UGIC was 55.47/100000 in Hebei Province, which was approximately 1.06 times higher than the rate in the Chinese population (52.45/100000) and far higher than the global rate (19.95/100000)[1,2]. In Hebei Province, newly diagnosed UGIC cases comprised 24.62% of all cancer types, whereas the cases accounted for only 10.0% of all cancer types worldwide. The mortality rate of UGIC in males in the rural areas of Hebei Province was approximately 5.08 times higher than the global statistics. Males in rural areas of Hebei Province were a population at high risk of developing UGIC.

Our data showed that the burden of UGIC was heavy in Hebei Province. UGIC resulted from lifestyle habits, such as an unreasonable dietary structure, tobacco smoking, alcohol drinking, smoked food consumption and poor oral health[13-17]. A significant inverse association has been reported between fruit consumption and UGIC (esophageal cancer, RR = 0.60; gastric cancer, odds ratio (OR) = 0.43)[18,19]. An intake of cereal fibre was significantly inversely associated with the risk of cancers of the gastrointestinal tract[18]. One recent study showed that insufficient consumption of fresh fruits may be a serious risk factor in Hebei Province, and the dietary fibre intake was only 37.3% of the reference intake from 2010-2013[20]. Heavy smoking and chronic alcohol consumption were reported to be major risk factors for UGIC[21]. People who were current cigarette smokers were associated with an increased risk of esophageal cancer (RR = 1.67) and gastric cancer (RR=1.60) compared with people who had never smoked[22]. In China, the smoking rate was 52.9% for males, and the second-hand smoking rate was approximately 72.4%, which was significantly higher than the global level[23]. In Hebei Province, the cigarette smoking rate was 26.08% for both genders and 48.09% for males[24]. Additionally, the smoking prevalence is still increasing. According to an investigation on alcohol consumption in adult residents in Hebei Province[25], the total drinking rate was 41.1% and was higher in males (71.8%) than in females (19.7%). Males were the primary population that drank alcohol. Because maintaining good life habits is necessary for health, we suggest that people, especially males in rural areas, quit smoking and limit alcohol consumption.

Economic, environmental and genetic risk factors can also lead to the high incidence and mortality rates of UGIC[26-28]. UGIC is a disease of the poor and the socially disadvantaged. A large number of epidemiological studies have confirmed that the UGIC risk is higher in populations with a lower socioeconomic status due to limited medical care, limited education and poor living conditions[29-33]. Almost 78% of the new cases occurred in rural areas of Hebei Province in 2013. The per capita annual net income of urban households (¥17278) was approximately 2.64 times higher than that of rural households (¥6539) in Hebei Province in the 2010s[34]. The low income sets constraints on the involvement of the population in health care for

the prevention or treatment of illnesses and injuries, the possibility of a good education level, which would improve their health awareness and lifestyle, the ability to live in a favourable environment where they could avoid exposure to bacteria, viruses and other infection-causing agents, and their dietary choices. One study identified risk factors for esophageal cancer in residents of Hebei Province and showed that living in rural areas (OR = 3.14) and eating mildewed food (OR = 7.44) were risk factors for esophageal cancer[35].

A family history of cancer is an important risk factor for UGIC. A first-degree family history of esophageal or gastric cancer was significantly associated with UGIC development, with an adjusted OR of 4.7 (95%CI: 2.6-8.4)[36]. A study on residents of Hebei Province found that having a family history of esophageal cancer was a risk factor for this malignancy, with an OR of 3.23[35]. In Cixian, a family history of gastric cancer was shown to be a risk factor for this malignancy (OR = 1.84)[7]. Therefore, appropriate screening strategies, especially for relatives of patients, should be considered for the prevention and control of UGIC in Hebei Province.

Moreover, UGIC was associated with geographic factors. Liang *et al*[8] showed that the nitrate, nitrite, nitrogen and ammonia levels in high-risk areas of Hebei Province were seven times higher than those in low-risk regions and exceeded the national standards for drinking water. Cao *et al*[6] also found that the nitrate level in Shexian (a high-risk area), Hebei Province was five times higher than that in Chicheng County (a low-risk area) and that the nitrogen and ammonia levels in Shexian were also higher. This finding suggests that nitrate and ammonia were important risk factors for UGIC. Therefore, we should improve drinking water quality and provide safe water to the high-risk areas of Hebei Province.

The mortality rate of UGIC in Hebei Province dropped from the 1970s to 2013. In Hebei Province, the data from the four periods of the death survey indicated that the mortality rate in 2013 had decreased by 20.42% compared with the mortality rate in 1973-1975 in the sampling survey of national causes of death. In both high-risk and non-high-risk areas, the mortality rates of UGIC dropped from the 1970s to 2013, although the rates declined more in the high-risk areas than in the non-high-risk areas.

The age-specific mortality rate was lower in 2013 than in 1973-1975, 1990-1992 and 2004-2005 for individuals younger than 75 years but was dramatically increased in the 75-79 age group. This latter group had an age-specific mortality rate in 2013 that was higher than the rates in 1973-1975, 1990-1992 and 2004-2005, showing the ageing population trend. At present, as economic growth drives the standards of living higher, the median age at death caused by UGIC increases. The median age at death caused by UGIC increased approximately 4 years from the 1970s to 2013. This rate is increasing faster in high-risk areas than in non-high-risk areas; indeed, the median age at death in high-risk areas (70.37) was basically the same as that in non-high-risk areas (70.40) in 2013. This finding indicated that we had achieved a great success in the screening, early detection and treatment of UGIC in Hebei Province, especially in high-risk areas. Additionally, diagnosis and treatment have improved, which can extend people's lifespan. However, we need to continue efforts to reduce the mortality rate.

The prognosis of UGIC is poor. The main reason is that most cases are asymptomatic during early stages and thus are detected at an advanced stage when they are no longer amenable to surgical resection. Therefore, screening and treating patients with upper gastrointestinal precancerous lesions is important for preventing the development of UGIC in high-risk individuals. Cixian and Shexian have been chosen as demonstration bases for the early detection and treatment of UGIC. Endoscopy with mucosal iodine staining is a sensitive technique to identify clinically relevant UGIC. Since 2000, a national screening programme using endoscopy with mucosal iodine staining and an index biopsy combined with a pathological examination to confirm and stage the disease has become available at 17 sites in Hebei Province, including Cixian and Shexian. At present, 25000 high-risk individuals in Hebei Province are screened every year. The screening programme has reduced the total cancer mortality rates in the Cixian and Shexian populations and may be one reason for the decreasing trend of mortality.

We showed the age, period and birth cohort effects of the condition in Cixian from 1989 to 2013 *via* an age-period-cohort model. The age and cohort effects played an important role. People aged between 65 and 69 years were a population at relatively high risk for UGIC. The total trend of the cohort effect was a decline, and the early birth cohort had higher risk of developing UGIC. One reason could be that long-term unhealthy lifestyle behaviours, such as heavy smoking, chronic alcohol consumption, and a weakened immune system, increased the birth cohort effect of the incidence risk in rural areas during these time periods. The period effect did not show large variation. Using the grey system model, we found that the mortality rates of UGIC in Cixian and Shexian exhibited a downward trend that should be attributed to the early diagnosis and treatment in these high-risk areas of Hebei Province. The model predicted that the mortality rates of UGIC would decrease to 98.80 and 133.99 per 100000 in 2018, respectively. However, we should still enhance UGIC control and prevention measures.

In summary, the mortality rate of UGIC has been decreasing over the past 40 years. However, UGIC was the major cause of cancer death in Hebei Province. Intensifying the primary prevention by adopting a healthy lifestyle and the secondary prevention with endoscopic iodine staining are priorities for the reduction of the morbidity and mortality of UGIC in

Hebei Province.

COMMENTS

Background

Globally, approximately 45% of upper gastrointestinal cancer cases (including esophageal cancer and stomach cancer) occur in China. The incidence and mortality proportions of these cancers account for approximately 19.8% and 23.3% of all malignancies in China, respectively. Hebei Province is recognized as a high-risk area for upper gastrointestinal cancer.

Research frontiers

In China, the burden of upper gastrointestinal cancer is heavy. However, few studies have investigated the epidemiological trends of upper gastrointestinal cancer. This research focused on the analysis of the real burden of upper gastrointestinal cancer in Hebei Province and the trend in the high-risk regions of Hebei Province and China to provide epidemiological evidence for developing strategies to control upper gastrointestinal cancer.

Innovations and breakthroughs

The study collected data from 21 population-based cancer registries covering 11185626 individuals in Hebei Province in 2013. The authors applied the age-period-cohort model to analyse the incidence rate of upper gastrointestinal cancer in one high-risk area of Hebei Province (Cixian), and predicted the mortality rates of upper gastrointestinal cancer in the high-risk areas of Hebei Province (Cixian and Shexian) using a grey system model.

Applications

This study investigated the real burden of upper gastrointestinal cancer in Hebei Province. This study analysed the reasons why the incidence and mortality rates of upper gastrointestinal cancer were higher in the high-risk areas than in the non-high-risk areas and why the mortality rates decreased. The results will provide reference values for upper gastrointestinal cancer control.

Peer-review

The aim of this study was to provide epidemiological evidence for developing strategies to control upper gastrointestinal cancer (UGIC), which is defined as the principle cause of death in a large area of China(*i.e.,* Hebei Province, which has a risk that is 5.08 times higher than the estimated global risk). All of the epidemiological data are of enormous importance since they represent the basis for a smart health policy that should be oriented and balanced on actual risk data for different regions and populations. The manuscript is well written and organized.

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