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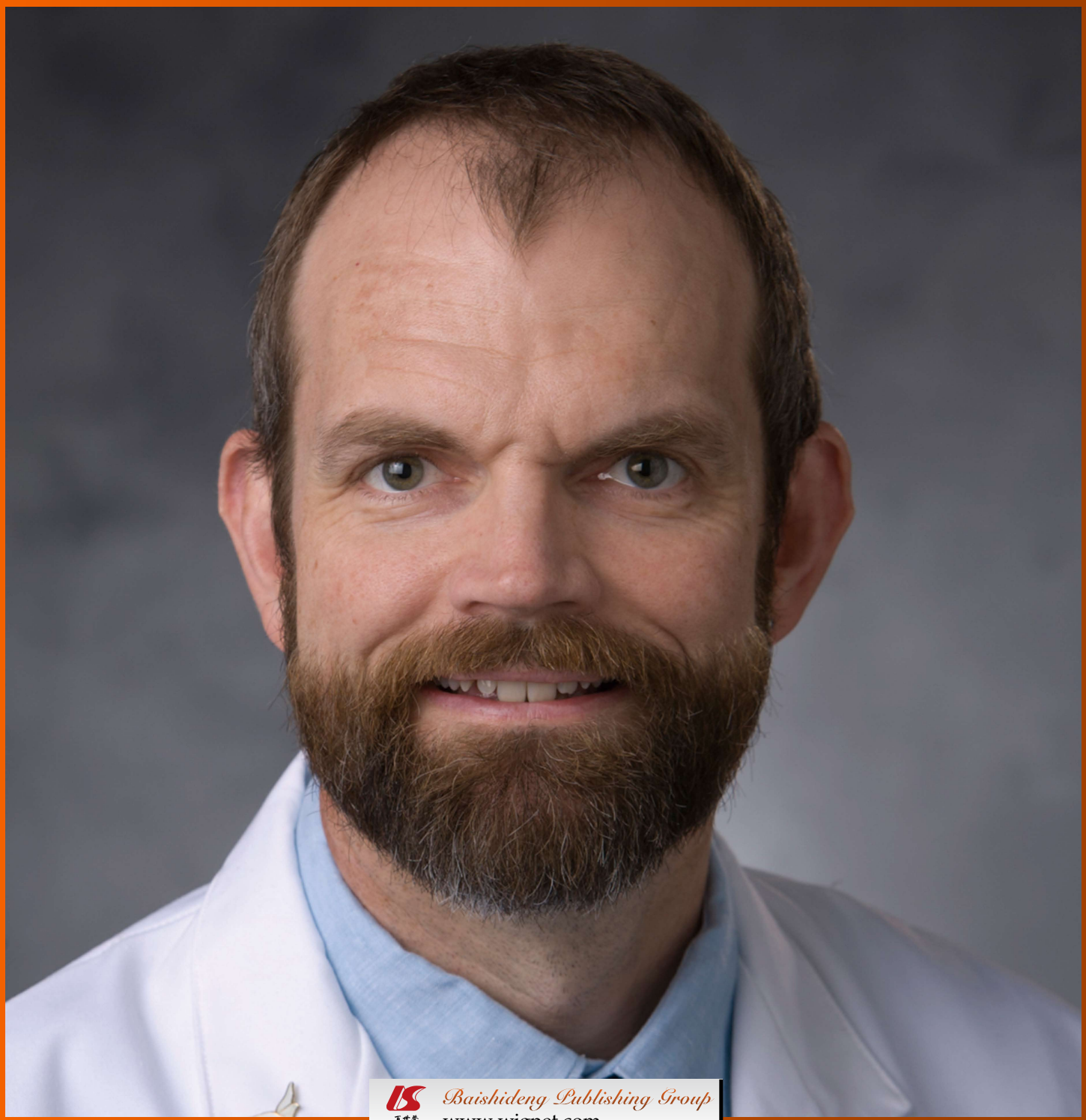

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EDITORIAL

Confocal laser endomicroscopy in inflammatory bowel diseases: Dream or reality?

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Abstract

Confocal laser endomicroscopy (CLE) is a newly introduced procedure that provide real-time, high-resolution imaging of the gastrointestinal mucosa during endoscopy, allowing the visualization of the pathology of the mucosal epithelium with its cellular and subcellular structures. Recently, the use of CLE was reported in the study of colonic mucosa in patients with inflammatory bowel diseases and in particular in patients affected by ulcerative colitis. CLE has the potential to have an important role in management of inflammatory bowel diseases (IBD) patients as it can be used to assess the grading of colitis and in detection of microscopic colitis in endoscopically silent segments. Moreover, CLE can be used in surveillance programs especially in high-risk patients. Finally, CLE has been effectively used in diagnosing a biliary dysplasia/neoplasia in patients with primary sclerosing cholangitis, a pathological condition frequently associated with IBD, with a coexisting bile duct stricture.

Key words: Inflammatory bowel diseases; Endoscopy; Confocal laser endomicroscopy; Colon cancer; Dysplasia

Core tip: This paper reviews the current data on the clinical application of confocal laser endomicroscopy (CLE) in the study of colonic mucosa in patients with inflammatory bowel diseases (ulcerative colitis and Chron's disease). Moreover, the use of CLE has in diagnosing a biliary dysplasia/neoplasia in patients with primary sclerosing cholangitis, is evidenced.

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CONFOCAL LASER ENDOMICROSCOPY

Confocal laser endomicroscopy (CLE) is a newly introduced procedure which allows to capture the images of "virtual histology" of the gastrointestinal mucosa during endoscopy[1,2], so offering the opportunity to get the "real time" visualization of the pathology of the mucosal epithelium with its cellular and subcellular structures[3,4]. At present, CLE can be performed with 2 devices: one integrated into an endoscope (Pentax, Tokio, Japan, herein termed e-CLE) and one as a mini-probe through the scope (p-CLE; Cellvizio, Mauna Kea Technologies, Paris, France). Confocal microscopy consists of focusing a laser ray onto the mucosal surface and filtering the returned light by means of a small pinhole which rejects out-of-focus light. The illumination and detection systems are in the same focal plane and are termed "confocal". After passing the pinhole, the fluorescent light is detected by a photo-detection, transforming the light signal into an electrical one that is recorded by a computer. All detected signals from the illuminated spot are captured and measured. As the laser scans over the plane of interest, a whole

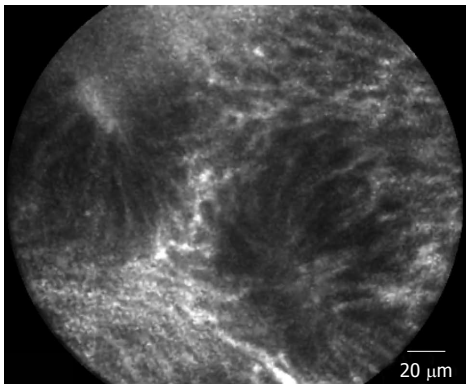

Figure 1 Rectal mucosa of patient in remission from ulcerative colitis. Irregular alignment of crypt, crypt distortion and fusion with reduced amount of goblet cells.

image is obtained pixel-by-pixel and line-by-line, whereas the brightness of a resulting image pixel corresponds to the relative intensity of detected fluorescent light. The gray-scale image created is an optical section representing one focal plane within the examined specimen. Real-time confocal laser scanning microscopy-sequences (duration 1 min are recorded and stored digitally for later evaluation. CLE evaluation and its relative high-quality images have shown high agreement with the real histology of the tissue, so opening a wide spectrum of potential applications, all focused on the possibility of reducing and/or targeting the biopsies during endoscopy[5,6]. The current potential indications for CLE imaging are broad and include almost all the cases in which endoscopic biopsy is needed.

At now, various studies have addressed the potential usefulness of CLE in diagnostic work-up of inflammatory bowel disease (IBD), with particular interest to ulcerative colitis (UC)[7-9]. In effect, all the studies concerning the application of CLE in the UC context have shown that this technique can have a potential role in assessing the extension and the activity of disease and in targeting biopsies, reducing the number of useless biopsies and improving the early detection of dysplasia[10-13]. In the field of UC, the most frequent alterations in crypt architecture are represented by dilation of crypt openings, more irregular arrangement of crypts, enlarged spaces between crypt, crypt destruction and/or crypt fusion, and crypt abscess with fluorescein leaks into the crypt lumen (therefore making the lumen brighter than the surrounding epithelium). Microvascular alterations are mainly represented by dilated, prominent branching vessels (Figures 1 and 2). The study by Watanabe *et al*[14], including 17 patients with active UC compared with 14 controls, showed that CLE images provided equivalent information to histopathology with respect to definition of the main histological outcomes (crypt architecture, capillaries and inflammatory cells). On these bases, a new classification of inflammatory activity in UC using CLE has been proposed[15], which comprises the assessment of crypt architecture, microvascular alterations and fluorescein leakage.

One of the most important diagnostic goals in the management of patients with UC, especially of those who present risk factors for cancer development, should be the "real-time" endoscopic identification and diagnosis of dysplasia/neoplasia, as this would reduce the number of unnecessary biopsies with their associated time and costs[16,17]. Starting from these assumptions, Kiesslich *et al*[18] have shown for the first time that the diagnosis of dysplasia/neoplasia in UC could be maximized by using both pan-chromoendoscopy (CE) and targeted CLE, with high values of diagnostic accuracy (sensitivity 94%, specificity 98%). This result has been recently confirmed, although with less remarkable diagnostic values, by van den Broek *et al*[19], who reported a diagnostic accuracy of 81% when comparing CLE with narrow-band imaging plus high-definition endoscopy (diagnostic accuracy 92%). In accordance with these reports, a recent paper produced by our group, exploring the efficacy of the combined application of CE and targeted p-CLE in diagnosing dysplasia in longstanding UC in the "real-life", has underlined the high diagnostic accuracy of such a procedure compared to standard histology (sensitivity 100%, specificity 90%, positive predictive value 83% and negative predictive value 100%)[20] (Figure 3). Giving value to all the above-mentioned contributions, the combination of CE and CLE corresponds to a diagnostic gain of 3- to 5-fold for detecting dysplasia/neoplasia than conventional colonoscopy[21]. The diagnostic gain is mainly due to CE application which could dramatically decreased (about of 10 times) the number of biopsies when just circumscribed suspicious lesions on CE would have been targeted; if only CLE-suspected neoplastic lesions had undergone biopsy after CE, the mean number of biopsies would be further reduced.

More recently, Neumann *et al*[22] have explored the clinical utility of CLE also in 76 patients affected by Crohn's disease (CD), particularly determining whether the disease activity can be graded by using CLE. In effect, a relevant percentage of patients with active CD presented an increased colonic crypt tortuosity, enlarged crypt lumen, microerosions, augmented vascularization and increased cellular infiltrates. Starting from these considerations, these authors proposed a CLE score for assessing CD activity in vitro, with such a score having of potential utility for predicting the course of CD and the response to medical therapy.

CLE application in IBD has been evaluated even under a prognostic view. A nice work by Kiesslich *et al*[23] have shown that "cell shedding" and "barrier loss" detected by CLE are able to predict relapse of IBD and have potential role as diagnostic tool for the management of the disease. In this paper, the sensitivity, specificity and accuracy for the "CLE grading system" to predict a flare were 62.5%, 91.2% and 79%, respectively. Interestingly, a recent paper by Turcotte *et al*[24] confirmed the high prognostic power of CLE in predicting the course for other relevant clinical end-points for patients affected by IBD.

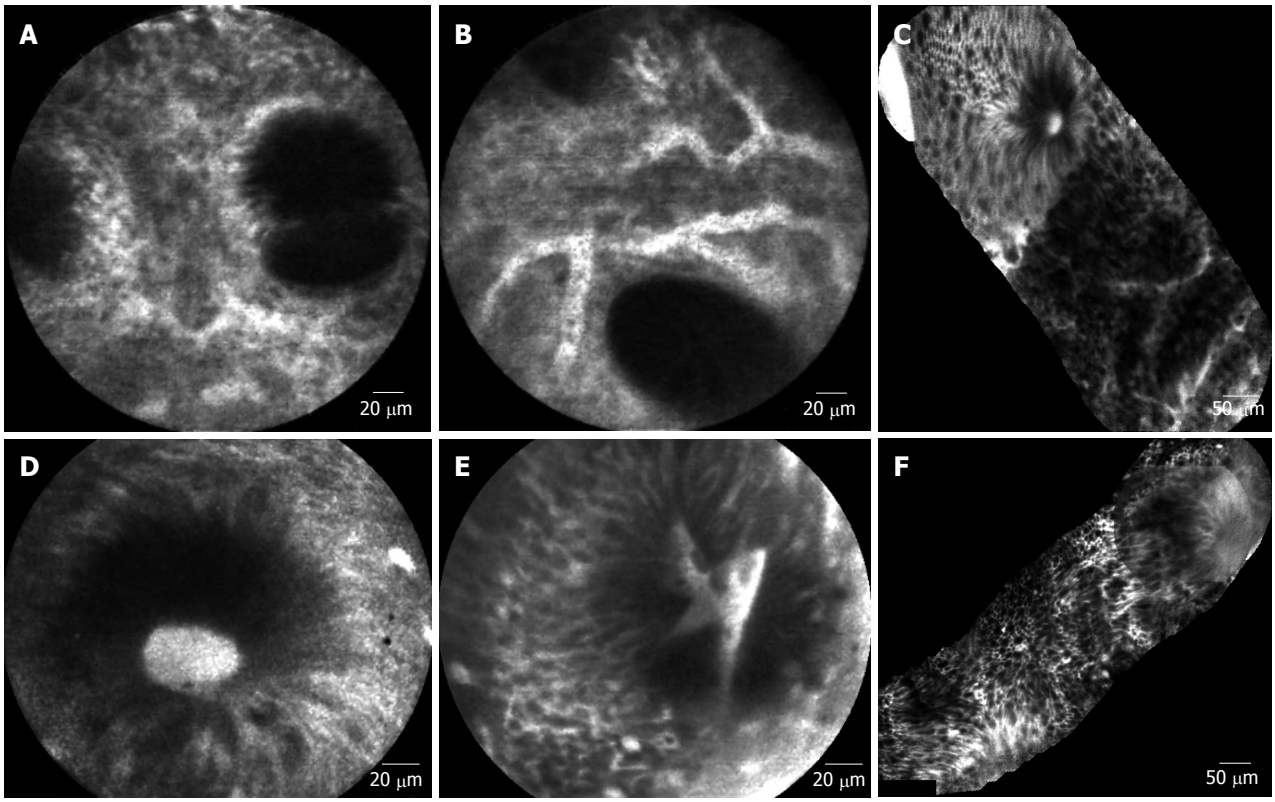

Figure 2 Colonic mucosa. A: Patient in remission from ulcerative colitis. Crypt distortion and fusion and many capillaries visible in the lamina propria; B: Patient in remission from ulcerative colitis. Enlarged spaces between crypts and dilated prominent branching vessels; C: In patient with active ulcerative colitis (distal colitis). Image of colonic mucosa showing the switch from normal mucosa (top of the figure) to inflamed mucosa Inflamed mucosa showing irregular arrangement of crypts, crypt fusion and capillaries alterations; D: In patient with active ulcerative colitis. Dilated and bright crypt lumen (fluorescein leakage) with intact epithelium; E: In patient with active ulcerative colitis. Dilated, irregular and bright crypt lumen (fluorescein leakage) with partially intact epithelium; F: In patient with highly active ulcerative colitis (Mayo CU3). Crypts distortion and destruction, crypt abscess and crypts replacement by diffuse necrosis.

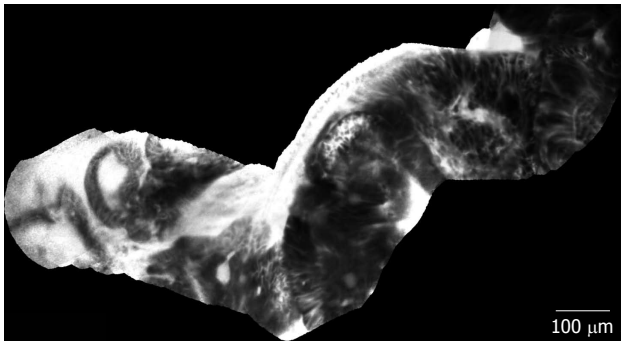

Figure 3 Dysplasia-associated lesional mass in long-standing ulcerative colitis. Image of colonic mucosa evidencing the switch from the inflamed mucosa, to the neoplastic mucosa. Inflamed mucosa is characterized by crypts fusion and distortion, dilation of crypt openings, enlarged spaces between crypts, and microvascular alterations with fluorescein leaks into the crypt lumen. Dysplastic mucosa (right corner) is characterized by "dark" cells, irregular architectural patterns with villiform structures and a "dark" epithelial border.

In particular, increased epithelial gaps in the small intestine as determined by CLE were a predictive factor for future hospitalization or surgery in IBD patients.

Going to another potential indication of this procedure in diagnostic work-up of IBD patients, CLE has been effectively used in diagnosing a biliary dysplasia/neoplasia in patients with primary sclerosing cholangitis (PSC), a pathological condition frequently associated with IBD, with a coexisting bile duct stricture[25]. In effect, Heif *et al*[25] showed a high diagnostic accuracy in detecting the presence of a bile duct neoplasia in 15 PSC patients with 21 dominant stenoses (sensitivity 100%; specificity 61%; positive predictive value 22%; negative predictive value 100%). This paper has opened the doors to a further potential application of CLE in IBD.

Unfortunately, some relevant limitations reduced the current application of CLE in general practice: the need for a learning curve, the cost of the equipment, the need for an extra-time (about 30 min) to enhanced colonoscopy and, not less important, a number of medical-legal issues. Furthermore, the promising results in the literature derived from a little number of trials and still from a few experienced centers and, as a consequence, these cannot be generalized easily.

In our mind, among these limitations, the need for an adequate learning curve represents the less relevant topic. In effect, as shown in previous papers, the operator's endoscopic expertise and learning curve represent the crucial issues and main limitation for the routine application of this endoscopic technique. However, a recent report has highlighted that the ability to accurately interpret CLE images for predicting neoplastic lesions can be learned rapidly by a range of GI specialists[26]; similarly,

the ability to acquire high-quality CLE images can also be learned quickly.

About medical-legal issues, mainly regarding the application of CLE for surveillance endoscopy in UC, the principal matter is represented by the fact that endoscopists would make a histological diagnosis without the confirmation by a pathologist and would decide during the endoscopy if performing or not multiple biopsies. At now, this kind of diagnostic approach is not reported by the current guidelines[27] and should be applied only within a controlled trial formally approved by an ethical committee.

Concluding, new multicenter studies are needed to assess the real cost-effectiveness of CLE technique for IBD. In our opinion, when balancing the interesting diagnostic advantage of CLE in clinical practice with its important realistic limitation, the wide application of this procedure in the current endoscopic practice still appears to be more a dream then a reality.

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REVIEW

Epidemiology of esophageal cancer

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Abstract

Esophageal cancer (EsC) is one of the least studied and deadliest cancers worldwide because of its extremely aggressive nature and poor survival rate. It ranks sixth among all cancers in mortality. In retrospective studies of EsC, smoking, hot tea drinking, red meat consumption, poor oral health, low intake of fresh fruit and vegetables, and low socioeconomic status have been associated with a higher risk of esophageal squamous cell carcinoma. Barrett's esophagus is clearly recognized as a risk factor for EsC, and dysplasia remains the only factor useful for identifying patients at increased risk, for the development of esophageal adenocarcinoma in clinical practice. Here, we investigated the epidemiologic patterns and causes of EsC. Using population based cancer data from the Surveillance, Epidemiology and End Results Program of the United States; we generated the most up-to-date stage distribution and 5-year relative survival by stage at diagnosis for 1998-2009. Special note should be given to the fact that esophageal cancer, mainly adenocarcinoma, is one of the very few cancers that is contributing to increasing death rates (20%) among males in the United States. To further explore the mechanism of development of EsC will hopefully decrease the incidence of EsC and improve outcomes.

Key words: Risk factor; Barrett's esophagus; Cyclin D1 G870A; Esophageal neoplasm; Susceptibility; Polymorphism

Core tip: Here, we investigated the epidemiologic patterns and causes of esophageal cancer. Using population based cancer data from the Surveillance, Epidemiology and End Results Program of the United States; we generated the most up-to-date stage distribution and 5-year relative survival by stage at diagnosis for 1998-2009.

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INTRODUCTION

Esophageal cancer (EsC) including squamous cell carcinoma (SCC) and adenocarcinoma is considered as a serious malignancy with respect to prognosis and a fatal outcome in the great majority of cases[1,2]. Esophageal carcinoma affects more than 450000 people worldwide and the incidence is rapidly increasing[3]. Currently, EsC is the eighth most common incident cancer in the world because of its extremely aggressive nature and poor survival rate[4,5].

EsC exhibits an epidemiologic pattern distinct from all other cancers[6,7]. The incidence of esophageal adenocarcinoma has increased sharply over the past few decades, both by period and birth cohort. Etiological studies are required to explain the rapid increase of this lethal cancer[8]. Understanding the epidemiology of EsC will be the key to elucidating the causes and risk factors for esophageal cancer and thus the cornerstone of developing any prevention strategies.

PATHOLOGY AND ANATOMY

Cancer of the esophagus typically occurs in one of two

forms, SCCs arising from the stratified squamous epithelial lining of the organ, and adenocarcinomas affecting columnar glandular cells that replace the squamous epithelium[9]. Sarcomas and small cell carcinomas generally represent less than 1%-2% of all esophageal cancers[10,11]. On rare occasions, other carcinomas, melanomas, leiomyosarcomas, carcinoids, and lymphomas may develop in the esophagus as well[5].

SCC is the predominant histologic type of esophageal cancer worldwide[12]. The incidence of squamous cell cancer of the esophagus increases with age as well and peaks in the seventh decade of life. The incidence of squamous cell esophageal cancer is three times higher in blacks than in whites, whereas adenocarcinomas are more common in white men.

The natural histories of SCCs and adenocarcinomas of esophagus appear to differ substantially. For squamous cell cancers, transition models have described squamous epithelium undergoing inflammatory changes that progress to dysplasia and in situ malignant change[13,14].

Most adenocarcinomas, however, tend to arise in the distal esophagus from columnar-lined metaplastic epithelium, commonly known as Barrett's esophagus[15,16], which replaces the squamous epithelium during the healing reflux esophagitis and may progress to dysplasia. Gastroesophageal reflux disease (GERD), or just reflux[17-19] can damage the lining of esophagus which causes Barrett's esophagus[17], characterized by abnormal "tongues" of salmon-colored mucosa extending proximally from the gastroesophageal junction into the normal pale esophageal mucosa, develops in approximately 5 to 8 percent of patients with gastroesophageal reflux disease.

Cancers that start at the area where the esophagus joins the stomach (the GE junction), which includes about the first 2 inches of the stomach (called the cardia), tend to behave like esophagus cancers (and are treated like them, as well), so they are grouped with esophagus cancers. Approximately three quarters of all adenocarcinomas are found in the distal esophagus, whereas SCCs are more evenly distributed between the middle and lower third. The cervical esophagus is an uncommon site of disease. Nowadays the terminology used for the definition of adenocarcinomas at the GE junction is "cardiac carcinoma", which can be easily misunderstood. This definition of adenocarcinomas of the GE junction does not allow correct comparison of diagnosis (endoscopic, radiological and pathologic), epidemiology and surgical therapy in national and international aspects, because different tumor can develop in the same area, and all called cardia tumors[20]. Siewert and Stein recommended a classification to solve this problem[21]. The classification of the tumors is morphological/topographical[21,22]. Type I is adenocarcinoma of the distal part of the esophagus. Type II is adenocarcinoma of the real cardia and type III is subcardial gastric adenocarcinoma. The importance of this classification is it enables unified pre-operative assessment and it can also help to decide the type of the surgical intervention[20,23-27].

INCIDENCE

Cancers arising from the esophagus, including the GE junction, are relatively uncommon in the United States[28,29]. The rate of cancer of the distal esophagus is about equal to that of the more proximal two-thirds[30]. SCC is the predominant histologic type of esophageal cancer worldwide. The incidence of SCC increases with age as well and peaks in the seventh decade of life, which is three times higher in blacks than in whites, whereas adenocarcinomas are more common in white men.

The most important precancerous disease is Barrett's esophagus[31-34]. Patients with Barrett's esophagus have a 50 to 100 times increase in their risk of developing cancer compared to the general population. People with Barrett's esophagus are much more likely to develop cancer of the esophagus. These people require close medical follow-up in order to find cancer early. Still, although they have a higher risk, most people with Barrett's esophagus do not go on to develop cancer of the esophagus. In their population-based cohort study, Hvid-Jensen *et al*[35] reported an annual risk of esophageal adenocarcinoma of 0.12% among patients with Barrett's esophagus.

For different types of esophageal cancer, the risk increases with age, with a mean age at diagnosis of 67 years. Esophageal cancer age-adjusted incidence of blacks was about twice that of whites (8.63/100000 *vs* 4.39/100000, $P < 0.05$)[36]. Squamous cell carcinoma was more commonly diagnosed in blacks and white females, whereas adenocarcinoma was more common among white males.

Although the disease is relatively uncommon in the United States, it is a major global health threat[37]. Esophageal cancer is four times more common and slightly more lethal in men than in women. According to the National Cancer Institute (Cancer.gov) in 2012, it is estimated that 17460 persons (13950 men and 3510 women) will be diagnosed with and 15070 persons will die of cancer of the esophagus in 2012.

Esophageal cancer occurs at a rate 20 to 30 times higher in China than in the United States. An esophageal "cancer belt," primarily squamous cell cancers, extends from northeast China to the Middle East[38-40]. Evidence of an association between environment and diet and esophageal cancer comes from the profound differences in incidence observed in various parts of the world. The majority of the factors so far implicated in cancer of the esophagus appear to act directly on the esophagus rather than systemically. Nutritional deficiencies can develop by chronic alcohol use as well as by poverty and lack of an adequate food supply, but diet does not explain the whole picture. External carcinogens are necessary to affect the end result. The association between nutrition and esophagitis may suggest methods of primary prevention of esophageal cancer and provide a chance of lowering the incidence of this deadly disease[31].

From 1996-2009, the annual percentage change was increased by 0.5% in all races and 0.4% in white. How-

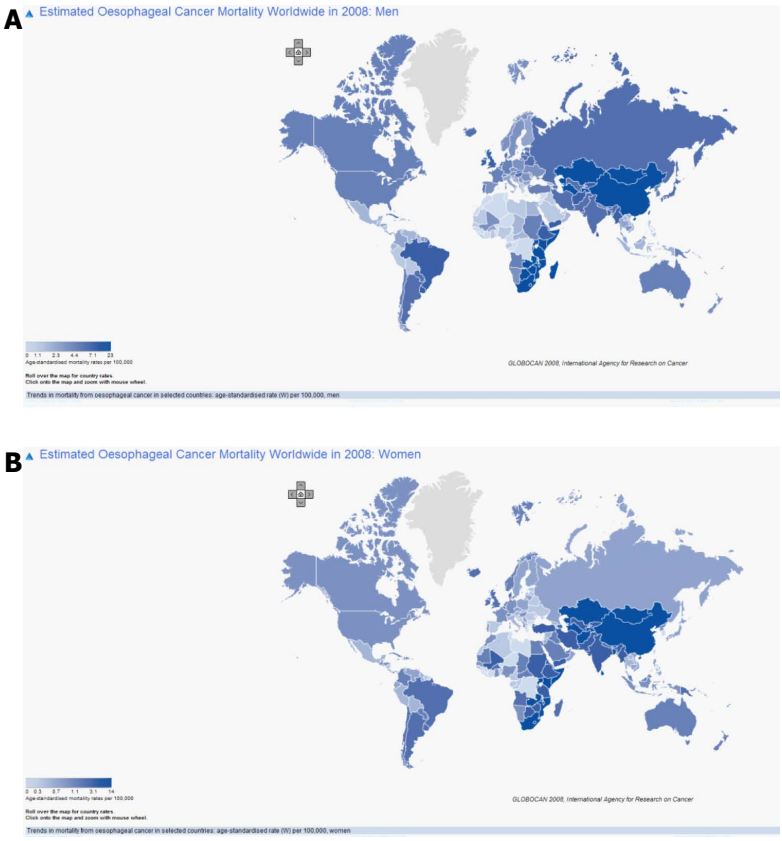

Figure 1 Estimated esophageal cancer mortality worldwide in 2008 (GLOBOCAN 2008). A: Men; B: Women.

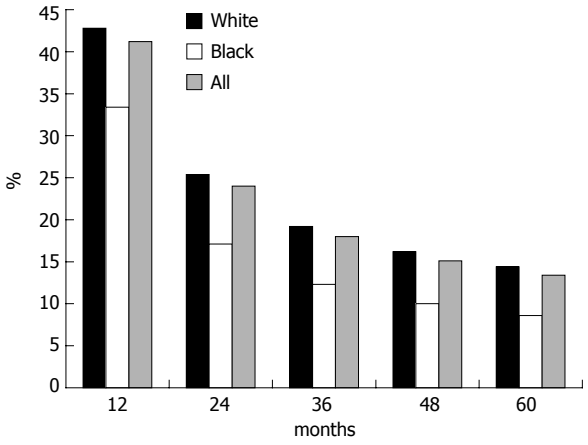

Figure 2 Relative survivals for esophageal cancer for all races.

ever, the increase of incidence is because of the increase incidence in men. Actually, the incidence in woman dropped by 0.4% (Surveillance, Epidemiology and End Results, SEER).

MORTALITY

Figure 1 shows the age-adjusted esophageal cancer mortality. It is in line with the incidence rate in the world but there is no difference between men and women. Age-adjusted mortality for blacks, although showing a declining trend, was nearly twice that of whites (7.79 *vs* 3.96, $P < 0.05$). Squamous cell carcinoma was more commonly diagnosed in blacks and white females, whereas adenocarcinoma was more common among white males ($P < 0.001$)[41]. The reasons are economic status, diet, and poor eating habits, *etc*.

SURVIVAL

Survival varied widely according to cancer site. The differences in survival related to histology were also expected[42]. Although survival was poor for all groups, it was significantly poorer in blacks than in whites (Figure 2). The overall 5-year relative survival for 2002-2008 from 18 SEER geographic areas was 16.9%. Five-year relative survival by race and sex was: 18.1% for white men; 17.0% for white women; 10.4% for black men; 12.6% for black women.

The overall relative 5-year survival rates over time increase gradually in white and black, man and women. For example, the rate was below 2% in 1995 to over 10% in 2008 in black men (SEER).

Although the overall outlook for patients diagnosed with esophageal cancer has improved in the past 30 years, most patients still present with advanced disease, and their survival remains poor[43]. One-third to one-half of patients treated with either chemoradiation therapy or chemoradiation therapy plus surgery are alive at 2 years, without recurrence of esophageal cancer.

The reason is because esophageal cancer is diagnosed at rather late stage. Overall, more than 30 percent of patients have metastatic disease at the time of presentation (32.15% in white and 31.83% in black). None was found that has in situ cancer, due to the fact that it can be diffi-

Table 1 Stage distribution and 5-year relative survival by stage at diagnosis for 1998-2009, all races, both sexes

Stage at diagnosis	Stage distribution	5-year relative survival
Localized (confined to primary site)	22%	37.80%
Regional (spread to regional lymphnodes)	30%	19.80%
Distant (cancer has metastasized)	35%	3.40%
Unknown (unstaged)	13%	10.50%

cult to diagnose esophageal cancer early. Among patients who are undergoing primary surgery, 22 percent have localized disease, 30 percent have regional cancer (Table 1).

RISK FACTORS

The patterns of esophageal cancer are dramatically changing in the United States. However, the mechanisms of esophageal tumorigenesis are not fully understood[5]. Three decades ago the large majority of these cancers were SCCs, but the incidence of esophageal adenocarcinoma has been steadily increasing[44]. Tobacco and alcohol consumption are the primary causes of SCCs of the esophagus[45]. One of the strongest emerging risk factors, however, is obesity. Increases in the prevalence of obesity and the incidence of esophageal adenocarcinoma are parallel, and several epidemiologic studies have shown upwards of threefold excess risks among overweight individuals. Further research into the causes of these usually fatal cancers may help identify other potential determinants and provide needed information to help stem their increase.

Cigarettes, red meat, alcohol and hookah smoking[4], nass use (a chewing tobacco product), opium consumption, hot tea drinking, poor oral health, low intake of fresh fruit and vegetables, and low socioeconomic status have been associated with a higher risk of esophageal SCC (Table 2). Barrett's esophagus is clearly recognized as a risk factor for EsC, and dysplasia remains the only factor useful for identifying patients at increased risk, for the development of esophageal adenocarcinoma in clinical practice.

Smoking increases risk of SCC and adenocarcinoma of the esophagus

Moderate to heavy smokers face an increased risk of both SCC and adenocarcinoma of the esophagus. Research suggests that when a smoker ingests tobacco condensates, it causes tobacco carcinogens, particularly nitrosamines, to come in contact with the esophageal mucosa. There is a direct correlation between the number of cigarettes a smoker smokes per day; the length of time the smoker spends smoking, and the risk of esophageal cancer[2].

The effects of chronic irritation and inflammation on SCC

The incidence of SCC of the esophagus has been found to dramatically increase in the presence of any factor that causes chronic irritation and inflammation, such as excessive alcohol intake, especially in combination with smoking[46,47]. This does not hold true for adenocarcinoma. This may account for more than 90 percent of all cases of SCC of the esophagus in developed countries[48].

Chronic esophageal irritation also occurs when food is retained and decomposed by bacteria, releasing various chemical irritants. Frequent consumption of hot beverages also appears to increase the incidence of SCC[49].

Table 2 Esophageal cancer risk factors[5,80-87]

Risk factor		Squmous-cell carcinoma	Adenocarcinoma
First or second hand smoke		+++	++
Alcohol consumption		+++	-
Consumption of red meat		+	+
Barrett's esophagus		-	++++
Reflux symptoms		-	+++
Being overweight		-	++
Poverty		++	-
Caustic injury to the esophagus		++++	-
History of head and neck cancer		++++	-
History with radiotherapy		+++	+++
Frequent consumption of extremely hot drinks		+	-
Polymorphism	Cyclin D1 (CCND1) G870A polymorphism	-	+
	p53 polymorphism	+	-
	TERT A279T polymorphism	+	+

-: No effect; +: Suspicious effect; ++: Positive effect; +++, ++++: Strong positive effect.

Obesity

Esophageal squamous cell carcinoma (ESCC) is clearly linked to a low socioeconomic status. The increasing prevalence of obesity in the Western world is thought to add to the rising incidence of esophageal adenocarcinoma. More specifically, it has been postulated that obesity increases intraabdominal pressure and gastroesophageal reflux by a specific mechanism, although some studies provided contradictory results. On the other hand, adipose tissue itself influences tumor development[50-54]. Adipocytes and inflammatory cells secrete adipokines and cytokines which are known to promote tumor development. The abundant availability of lipids from adipocytes in the tumor microenvironment, supports tumor progression and uncontrolled growth. Given that adipocytes are a major source of adipokines and energy for the cancer cell, understanding the mechanisms of metabolic symbiosis between cancer cells and adipocytes, should reveal new therapeutic possibilities.

Genetic changes

The genetic and molecular changes underlying the development of EsC remain poorly understood. Genetic analysis of these cancers reveals frequent chromosomal losses (4q, 5q, 9p, and 18q), chromosomal gains (8q, 17q, and 20q), and occasional gene amplifications (7, 8, and 17q)[5].

In the past decade, efforts have been made to use can-

didate gene approaches to identify genetic susceptibility factors for ESCC. The genome-wide association studies (GWAS) has emerged as a powerful and successful tool to identify common disease alleles by using high-throughput genotyping technology to interrogate a large number of tagging single nucleotide polymorphisms (SNPs) that serve as surrogates for untested common SNPs across the genome. So far, GWAS of esophageal cancers including ESCC in individuals of European and Japanese ancestry, have shown that variants in ADH genes and/or ALDH2 are associated with risk of ESCC[55-58]. More recently, Wu *et al* further reported that nine new ESCC susceptibility loci, of which seven, at chromosomes 4q23, 16q12.1, 17q21, 22q12, 3q27, 17p13 and 18p11, had a significant marginal effect ($P = 1.78 \times 10^{-39}$ to $P = 2.49 \times 10^{-11}$) and two of which, at 2q22 and 13q33, had a significant association only in the gene-alcohol drinking interaction [gene-environment interaction P ($P_{G \times E}$) = 4.39×10^{-11} and $P_{G \times E} = 4.80 \times 10^{-8}$, respectively]. Variants at the 4q23 locus, which includes the *ADH* cluster, each had a significant interaction with alcohol drinking in their association with ESCC risk ($P_{G \times E} = 2.54 \times 10^{-7}$ to 3.23×10^{-2}). They confirmed the known association of the *ALDH2* locus on 12q24 to ESCC, and a joint analysis showed that drinkers with both of the *ADH1B* and *ALDH2* risk alleles had a fourfold increased risk for ESCC compared to drinkers without these risk alleles. Their results underscore the direct genetic contribution to ESCC risk, as well as the genetic contribution to ESCC through interaction with alcohol consumption[55].

There are also some studies on polymorphism on other locations for esophageal adenocarcinoma with smaller samples. Cyclin D1 (*CCND1*) *G870A* polymorphism has been known to be a risk factor in multiple cancers[59-63]. However, investigations concerning the association of *CCND1 G870A* polymorphism with esophageal cancer risk have generated conflicting results[64-69]. The overall data suggest that CCND1 G870A variations might have an association with increased esophageal cancer susceptibility. The earliest findings, published in 2005, reported that CCND1 G870A was a risk factor for esophageal adenocarcinoma[67]. A study conducted by Liu's group, drew the exact opposite conclusion: CCND1 G870A was not associated with susceptibility to esophageal adenocarcinoma[70]. Liu's group explained the discrepancy by noting that all previous studies were based on small samplings.

Since the definition of G870A is the same for both groups, the significant difference lies in the methods that they used. Casson's group did polymerase chain reaction (PCR) followed by enzyme digestion, and visualized the result by running the products in a 15% acrylamide gel, and is referred to as "PCR-restriction fragment length polymorphism (RFLP)," which was widely used ten years ago. Liu's group genotyped by the 5'-nuclease assay (TaqMan), using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, United States). This is currently considered the gold standard in genotyping. Casson's group included patients with GERD. Since GERD is rather common in the general population, they selected strictly asymptomatic individuals for their control groups. Liu's group chose instead to use healthy visitors as their control group. And those healthy controls might have had some undiagnosed diseases related to GERD, such as Barrett's esophagus.

One source of bias between the two groups may lie in the different controls that were used. This may explain why the rate of G/G is different between the two groups. The second reason may be due to the detection method used. Usually, sequencing is viewed as the gold standard, but it is not always correct[71]. To detect polymorphism, the PCR-RFLP that Casson's group used, might have been a better choice because PCR-RFLP tests detect the correct genotype. Direct sequencing of the PCR products, obtained with one of the primers located adjacent to a mutated nucleotide, may cause unequal amplification of alleles in heterozygous samples. This effect is even stronger when mismatched primers are used. Therefore, there is a potential pitfall in DNA sequencing, indicating that sequencing may not always be the gold standard. The third reason may be due to the inherent differences between the two groups. As we know, the minor allele frequency (maf) of a SNP is different among different populations. Since it is ethnicity related, more information is needed to know the demographic information of the patient and the control group.

Although Li argues that others may be drawing different conclusions than his group, due to smaller samplings, it cannot be ruled out that other factors are involved, such as how the different control groups were recruited. Zhuo *et al*[64] reported that homozygous AA alleles might elevate esophageal cancer risk among Asians, but not Caucasians. This might partially explain why the two groups drew different conclusions.

CCND1 G870A polymorphism might be a low-penetrant risk factor for esophageal carcinoma, particularly among Asians. More information is needed to study large samples in relationship to pertinent demographic data.

PREVENTIVE FACTORS

The keys to prevention of esophageal cancer vary by cell type. For SCC, reduction or elimination of tobacco and alcohol consumption provide the best means to reduce the incidence of this cancer. However, no one particular risk factor is responsible for the rising incidence of esophageal adenocarcinoma. Several preventive strategies are under investigation using such agents as nonsteroidal anti-inflammatory drugs, selenium, alpha-difluoromethylornithine, and retinoids[72]. Vegetable intake, and fruit intake is considered to be a preventive role. Carotene, vitamin C, and vitamin E are protective, most likely in combination with each other and other micronutrients. The role of vitamin A is not clear because of conflicting findings in the studies reviewed[73]. When intake of raw vegetables and cooked vegetables was analyzed separately, raw vegetables were found to be more protective.

Because fruits are relatively expensive in most places, increased consumption may reflect higher socioeconomic status.

Since obesity is closely related to the incidence of the esophageal cancer, it would be interesting to follow up those patients with precancerous lesion to monitor their weight.

In patients with high-grade dysplasia, the options for preventive approaches include surveillance, endoscopic therapies, and surgical resection, but the optimum approach is debated[3]. In an analysis of more than 15 studies, the mean incidence of occult adenocarcinoma in patients with a preoperative diagnosis of high-grade dysplasia treated with esophagectomy was 41%. This high incidence provides a rationale for use of esophagectomy, but there is concern about the risk of morbidity. Use of endoscopic treatments for high-grade dysplasia has been supported in two randomised trials. In one trial of photodynamic therapy plus proton-pump inhibitors compared with proton-pump inhibitors alone, progression to cancer was significantly decreased in the photodynamic-therapy group (13% *vs* 28%). In the other, which assessed endoscopic radiofrequency ablation in patients with Barrett' s esophagus and high-grade dysplasia, radio frequency ablation was more effective in eradication of high-grade dysplasia than a proton-pump inhibitor alone, and the progression to cancer was lower (4% *vs* 22%) during short-term follow-up[74-77].

SCREENING AND EARLY DETECTION

Although several potential preventive measures exist, none has been proven to decrease the risk of esophageal carcinoma in prospective well-designed trials[3]. The relatively low incidence of esophageal cancer, the absence of early symptoms, and the rarity of a hereditary form of the disease make population-based screening untenable except in certain high-risk areas of the world[5].

Patients who are found to have Barrett's esophagus, however, may be candidates for regular endoscopic surveillance, since the incidence of low-grade dysplasia, high-grade dysplasia, and cancer is approximately 4 percent, 1 percent, and 0.5 percent per year, respectively, among such patients[5]. Whether endoscopic screening programs to detect Barrett's esophagus in patients with chronic reflux disease symptoms are useful has been debated. Critics point out the high number of people in the general population who have reflux symptoms and the fact that at least 40% of patients with Barrett's esophagus do not have reflux symptoms, and question the cost-effectiveness of screening. Proponents of screening for Barrett's esophagus point to the clear associations between reflux, Barrett's esophagus, and esophageal adenocarcinoma, and suggest that the rising incidence of esophageal adenocarcinoma justifies screening. No definitive data are available on whether endoscopic screening for Barrett's esophagus is associated with a reduction in cancer-related mortality and, therefore, screening is not routinely recommended.

However, some experts have recommended that endoscopy be performed every three to five years in patients who have Barrett's esophagus in the absence of epithelial dysplasia and more frequently if they are found to have low-grade dysplasia. Diagnostic endoscopy for early detection can be conducted in 2 steps: at first detection of an abnormal area through changes in relief, in color or in the course of superficial capillaries; then characterization of the morphology of the lesion. Then treatment decision offers 3 options according to histologic prediction: abstention, endoscopic resection, surgery. The rigorous quality control of endoscopy will reduce the miss rate of lesions and the occurrence of interval cancer[78].

CONCLUSION

The precise causes of EsC have not been identified. Despite uncertainties in our understanding of the causes of mechanistic pathways of esophageal cancer, there is sufficient evidence to take effective steps to prevent the majority of SCC in western countries, while more information is needed to curb the epidemic increase in adenocarcinoma[7,79].

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MINIREVIEWS

Appendectomy and *Clostridium difficile* colitis: Relationships revealed by clinical observations and immunology

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Abstract

Advances in understanding the interaction between the human immune system and the microbiome have led to an improved understanding of the function of the vermiform appendix as a safe-house for beneficial bacteria in the colon. These advances have been made despite long standing clinical observations that the appendectomy is a safe and effective procedure. However, more recent clinical data show that an appendectomy puts patients at increased risk for recurrent *Clostridium difficile* (*C. difficile*)-associated colitis, and probably other diseases associated with an altered microbiome. At the same time, appendectomy does not apparently put patients at risk for an initial onset of *C. difficile*-associated colitis. These clinical observations point toward the idea that the vermiform appendix might not effectively protect the microbiome in the face of broad spectrum antibiotics, the use of which precedes the initial onset of *C. difficile*-associated colitis. Further, these observations point to the idea that historically important threats to the microbiome such as infectious gastrointestinal pathogens have been supplanted by other threats, particularly the use of broad spectrum antibiotics.

Key words: Appendectomy; *Clostridium difficile*; Colitis; Diarrheal illness; Vermiform appendix

Core tip: Although the function of the appendix has remained an enigma for centuries, recently emerging advances in the fields of immunology and gut microbiology have merged with observations made in the clinic to form a coherent picture. Although the appendix is apparently a safe-house for beneficial bacteria, it seems likely that this safe-house does not satisfactorily protect the microbiome from broad spectrum antibiotics. In this view, selection pressures which threatened the microbiome and likely drove the evolution of the appendix have been supplanted in post-industrial society by new threats to the microbiome that the human body is not adapted for.

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INTRODUCTION

Is appendectomy the removal of a functional organ?

Appendectomy, like a wide variety of other surgical procedures, is extremely common in industrialized society. However, unlike common surgical procedures that include sterilizations for contraception, Cesarean sections, and inguinal hernia repairs, appendectomies are frequently performed as a prophylaxis for disease. The lifetime risk for appendicitis is only 8.6% for males and 6.7% for females, contrasting to the 12% and 23% lifetime rate of appendectomies performed, respectively[1]. These numbers indicate that approximately half of all appendectomies, including more than 60% in females, are

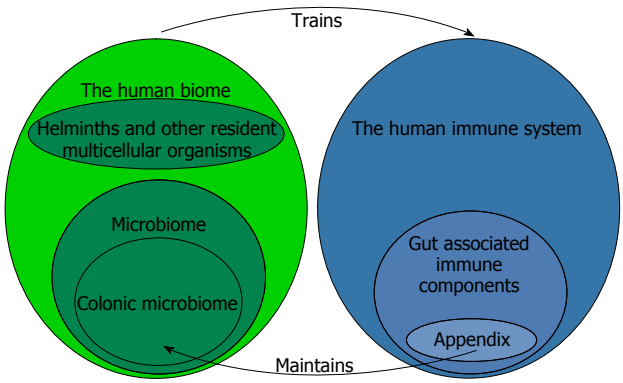

Figure 1 Interactions between the human biome (left Venn diagram) and the human immune system (right Venn diagram). In this view, all living organisms associated with the human body, either as permanent residents or through transient interactions, are part of the human biome. Two subsets of the biome, helminths and the microbiome, are shown as being part of the human biome. The colonic microbiome, in turn, is shown as being a subset of the microbiome in the Venn diagram of the human biome. Similarly, the appendix is shown as being a subset of the gut associated immune components, which in turn are a subset of the entire human immune system. The idea that the biome "trains" the immune system, equivalent to the view that the immune system is dependent on the biome for proper development, is illustrated. In this model, profound alterations in the biome as a result of post-industrial societies lead to aberrant immune system development, resulting in a variety of immune related pathologies, including appendicitis. The view that the appendix assists in maintaining of the colonic microbiome is also shown. Alterations in the biome that affect immune system training in post-industrial societies predominantly involve compartments of the biome other than the microbiome (*e.g.*, loss of helminths), so the processes leading to appendicitis are generally distinct from processes involved in support of the microbiome by the appendix.

incidental procedures, aimed at averting future episodes of appendicitis. This approach is generally successful, but 36 incidental appendectomies are required to prevent one case of appendicitis[1]. Given the large number of appendectomies currently performed, many of them elective, recently emerging evidence regarding the apparent function of the vermiform appendix has justifiably garnered much interest.

The idea that the vermiform appendix is a vestige of evolution was developed more than 150 years ago by Darwin[2]. The proposal was simple and made sense in the light of available data: the appendix and small cecum present in humans and some primates is the remainder of a larger cecum used for fermentation in a human ancestor with a diet much higher in fiber[2]. However, recent studies using current methods employed in the field that Darwin[2] founded have disproved that idea. In summary, a modern cladistics-based approach demonstrates that the appendix has evolved repeatedly in a wide range of animals, that some clades have a propensity to evolve an appendix, and that the evolution of the appendix is usually not associated with a decrease in the size of the cecum. In fact, a recent analysis of 361 mammalian species found a significant direct correlation between appendix and cecum size[3]. In other words, the appendix tends to be associated with a large cecum, not a smaller one. At present, many questions regarding the evolution of the appendix remain unanswered: it is not even known whether the first appendix evolved before or after the first cecum[4], or how often which precedes the other in evolution (given the rise of the appendix more than once during evolution). Although the absolute disproof of Darwin's views of the appendix is recent, the idea that the appendix is a vestige of evolution has been disputed effectively for more than a century. For example, Berry[5] concluded in 1900 that, based on anatomical and phylogenetic data, "The vermiform appendix of man is not, therefore, a vestigial structure. On the contrary, it is a specialized part of the alimentary canal". Keith[6] supported Berry's views and argued further that that the appendix, rather than being a flawed structure which gives rise to appendicitis, is a victim of changes in the environment due to industrialization: "When we come to realize how slowly evolutionary processes have affected man's body in past times, we can hardly expect our internal digestive system to adapt itself to the rapid pace demanded by the ever-accumulating resources of civilization".

When Keith[6] recorded his views in 1912, the incidence of appendicitis had profoundly increased in the lifetime of many practicing physicians, and it was therefore correctly surmised that something environmental was causing the disease. The opinion of the day was that changing diet following industrialization was in some way responsible for appendicitis. Although the view that appendicitis was due to an environmental factor or factors in industrial and post-industrial environments was solidified by numerous epidemiologic studies[7-11], it was not until the 1980s that Barker *et al*[12-14] determined that factors associated with indoor plumbing were somehow responsible for appendicitis[14]. These intriguing findings by Barker as well as additional work by Strachan on allergic disease[15] eventually gave rise to the currently held view that factors within post-industrial culture, including sanitation practices (*e.g.*, toilets and water treatment facilities) and modern medicine, lead to depletion of species normally associated with the ecosystem of the human body, or the "human biome" (not to be confused with the "microbiome", Figure 1). The resulting state, termed "biome depletion" is associated with a profoundly overreactive immune system that is prone to a variety of immune related diseases, including appendicitis. Barker's personal view is that the introduction of running hot water into a home might be the single most telling factor associated with an increased incidence of appendicitis (personal communication to Parker W). Since hot water is necessary for the effective use of soap, and given the effectiveness of soap in biological decontamination, this view makes sense. Here it should be noted that approaches which deal with the consequences of biome depletion are expected to one-day make appendicitis a rare disease. These approaches involve reconstitution of the human biome without abandoning the modern technology, including soap, water treatment facilities and medicine, which so effectively prevents the spread of water-borne disease[16-18].

Despite proof that the appendix is not a vestige of

evolution and that appendicitis is not the result of a faulty structure, the idea that the appendix is a vestige seems attractive simply because removal of the appendix does not, to the practicing physician or to the patients concerned, seem to have deleterious effects. This observation, apparent to everyone, presents a quandary: how can the appendix have some function, but yet appendectomy has no negative side effects? The answer to this quandary is readily apparent if one considers that actual function of the appendix.

In 2003 it was observed that the immune system apparently supports growth of mutualistic biofilms in the mammalian gut[19]. This view, although surprising at the time due to prevailing views in the field of immunology, now seems rather obvious in hindsight based on current knowledge regarding microbial ecology and host-microbe relationships[20-22]. This new view led to the evaluation of biofilm distribution in the human gut, and biofilms were indeed found to be most abundant in the appendix, where immune tissue had long been known to be the most abundant within the gut. This biofilm distribution in the gut set the stage for a deductive proof regarding the function of the appendix: Since the appendix is a structure harboring microbial biofilms, and since biofilms are protective of bacteria (a long standing observation in the field of microbiology), the appendix is, in essence, a safe house for bacteria (Figure 1). Given the shape and location of the appendix, it would indeed be difficult to imagine how the appendix might not be protective of bacteria.

Given the apparent function of the appendix, it has been proposed that an evolutionary driving force for the emergence of the appendix may be as an aid in the recovery from diarrheal illness associated with gastrointestinal (GI) infection. In this view, fragments of biofilms routinely shed from the appendix would serve as "seeds" for inoculation of the colon with a normal microbial flora following a diarrheal purge[23]. This explanation makes sense in light of (1) the relative seclusion of the apex of the appendix from the fecal stream, which presumably affords some protection from pathogenic organisms that might temporarily infect the GI tract; and (2) the pronounced role of diarrheal illness in human survival. Indeed, water-borne diseases followed by dysentery are frequently the leading cause of death during war and natural disasters[24-27], have affected both the rich and the poor[28] and are still one of the leading causes of death in developing cultures[29,30]. These observations are consistent with the view that that rapid reconstitution of the microbiome and restoration of a normal bowel following diarrheal illness might be adaptive in many circumstances. In fact, the relatively low mortality rate associated with diarrheal illness, less than one percent[31], is possibly a testament to the effectiveness of natural recovery mechanisms such as those that might involve the appendix. Adding further weight to this view, a very recent study by Guanine *et al*[32] found that "the human appendix contains a wealth of microbes, including members of 15 phyla". Species identified included members of phyla which constitute more than 98% of the normal colonic microbiome (*Firmicutes*, *Proteobacteria*, *Bacteroidetes Actinobacteria*, and *Fusobacteria*), indicating that the appendix possesses a microbial diversity sufficient to reconstitute the microbiome of the colon.

If this inductive rationale is correct, the paradoxical removal of the functional appendix without immediate and substantial harm is readily explained: Although water-borne disease is one of the leading causes of death in developing countries, the use of modern water treatment facilities and sanitation prevents widespread outbreaks of pathogens which might deplete the normal flora from a substantial portion of the population. Further, the absence of starvation and the presence of modern medicine in developed countries minimize the effects of diarrheal illness on the population.

Causes of appendicitis

Approximately 50% of cases of appendicitis are generally considered to be enigmatic in origin, with the remainder being attributed to a blockage of the appendix. However, work from David Barker during the 1980's first identified clues which eventually pointed toward the underlying cause of appendicitis. Barker noticed that epidemics of appendicitis followed the introduction of indoor plumbing into various communities. This observation was followed by epidemiologic studies showing that appendicitis is associated with developed but not with developing countries. Almost at the same time, another epidemiologist, Strachan[15], found that a hyper-active immune system is a consequence of the hygienic environment following the industrial revolution[15]. Strachan's observations point toward the idea that appendicitis, like many other allergic, autoimmune, and inflammatory diseases, is a result of biome depletion, a consequence of industrialization[16-18]. This culture-related basis for appendicitis explains why the appendix was not selected against during the course of evolution. Many components of the immune system, such as the appendix, are made obsolete by post-industrialized society, and these have also not been selected against during evolutionary history. Not only are these components now obsolete, but these components often become overly sensitive due to an absence of stimulation and cause detrimental health effects, such as ulcerative colitis that is exacerbated by the appendix[33]. Another example of a maladapted immune component is the immune compartment that produces immunoglobulin E (IgE). High levels of IgE lead to allergies and other destructive side effects in industrialized societies, but levels significantly higher than those found in industrialized countries are present in developing countries as a result of productive (beneficial) responses to parasitic infections[34-36].

THE EFFECT OF APPENDECTOMY IN LIGHT OF THE FUNCTION OF THE APPENDIX

Although an appendectomy is a relatively simple surgical procedure, the effects of removing the appendix are not necessarily straightforward. The appendix is associated with the highest concentration of gut associated lymphoid tissue (GALT) in the gut, and the function of the GALT is vastly complex and incompletely understood. Thus, an appendectomy is expected to profoundly alter the immune system with its hundreds or possibly thousands of interconnected components. Numerous functions have been attributed to the GALT, and it remains unknown how appendectomy alters many of those functions. However, some effects are established. First, appendectomy does have a moderating effect on pathogenic inflammatory immune responses of the gut. The observation that patients without an appendix tend to be at less risk for ulcerative colitis is more than 10 years old[33]. More recently, Bolin *et al*[37] used appendectomy as a treatment for ulcerative proctitis, a form of colitis, and showed an improvement of symptoms in 90% of patients, with complete remission in 40% of patients[37]. Possibly the most straight-forward explanation for this result is that removal of a substantial amount of GALT from the intestinal tract led to decreased immune reactivity in the gut. Whether the "safe-house" function of the appendix had anything to do with the result seems more speculative.

The appendix and the initial onset of Clostridium difficile colitis

Perhaps the most intriguing effects of appendectomy involve its effects on the incidence of *Clostridium difficile* (*C. difficile*) colitis. *C. difficile* colitis is a pathogenic state associated with overgrowth of the bacterium *C. difficile*, a gram-positive, spore-forming, anaerobic bacillus[38,39], and is generally not seen in individuals with a normal microbiome. However, alteration of the normal flora (generally by antibiotic use) can lead to overgrowth of *C. difficile* and subsequent disease. Recurrent *C. difficile* colitis is not a minor problem in modern medical practice, with one study showing nosocomial *C. difficile* diarrhea present in 3.4 to 8.4 cases per 1000 hospital admissions[40], and an increase in in-hospital mortality from 2.4% to 13.5%[41].

It might at first glance be expected that the appendix, if present, would be protective against *C. difficile* overgrowth. There is, however, at least one central problem with this supposition: it remains unknown if the appendix can effectively protect mutualistic bacteria against the modern antibiotics which generally precede *C. difficile* colitis. It seems reasonable that the appendix has evolved in the presence of enteric pathogens and thus that it may be effective in helping the body to recover from infectious disease. However, the use of high dose antibiotics is a very recent development in human history, and thus it is not reasonable to assume that the appendix may be protective under these conditions. To our knowledge, no studies have addressed this issue. Our laboratory has assessed the protection from antibiotics afforded by immune-mediated biofilms *in vitro*, and found that immune mediated biofilms formed by one species (*Escherichia coli*) are poorly protected from antibiotics. However, much additional work needs to be done in this field using a wide range of microbial species as well as whole animal models before any sort of answer which might have clinical implications can be obtained.

The supposition that the appendix, if indeed it is a safe-house for bacteria, should be protective against *C. difficile* colitis has a second potential flaw: If indeed the appendix does not protect mutualistic bacteria from antibiotic use, the appendix could hypothetically protect those organisms which are resistant to antibiotics, such as *C. difficile*, from a diarrheal purge. Thus, if the appendix performs its function perfectly, it could hypothetically increase the incidence of *C. difficile* colitis in the face of antibiotic use. Fortunately, this does not appear to be the case. At present, clinical data point toward the idea that the presence or absence of an appendix does not strongly affect the propensity for the initial onset of *C. difficile* colitis. In a study by Im *et al*[42], 80% of their patients with *C. difficile* colitis (203 out of 253) had an appendix, which is only slightly lower than the percentage found in the total population[1]. Another study, by Merchant *et al*[41], obtained essentially identical results, with 80% of their patients with *C. difficile* colitis (109 out of 136) having an appendix. Merchant *et al*[41] found that 82% of "normal" individuals (in their study, patients without GI complaints) had an appendix, as would be expected based on larger studies[1]. However, these observations do not directly address the actual effect of the appendix on the propensity for *C. difficile* colitis following antibiotic use, since they do not address the effect of appendectomy on the use of antibiotics. In other words, the data indicate that appendectomy does not affect the risk for *C. difficile* colitis, but it does not indicate whether an appendectomy might affect the risk for *C. difficile* colitis following antibiotic treatment. Since Merchant *et al*[41] did not control for antibiotic treatment, increased antibiotic use in those with an appendix, if it exists, would have confounded the study. Nevertheless, the observations do clearly indicate that the loss of an appendix is not associated with a dramatically increased risk for an initial onset of *C. difficile* colitis.

As stated above, it is possible that a perfectly functional appendix, if indeed it did not protect the normal flora from antibiotics, might selectively protect antibiotic resistant organisms such as *C. difficile* from a diarrheal purge. This possibility has been previously proposed by Merchant *et al*[41]. However, since the relative number of patients with and without an appendix in patient groups with *C. difficile* colitis is essentially the same as that in the normal population, the possibility that the appendix preferentially protects *C. difficile* seems extremely unlikely. Further, appendectomy itself affords a much lower risk of *C. difficile* colitis (0.2%) compared to colectomy (1.11%)

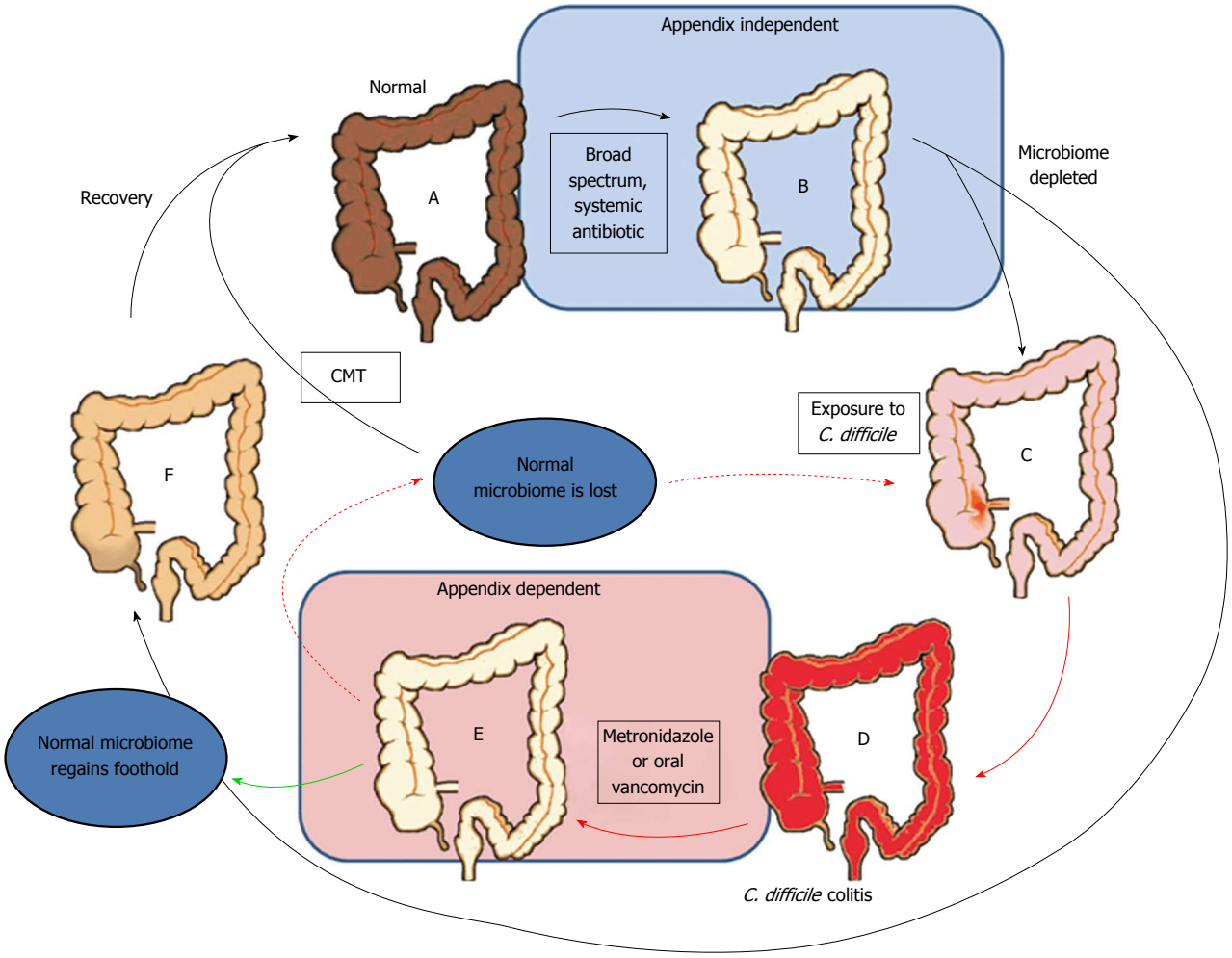

Figure 2 The cycle of microbiome depletion with antibiotics, the occurrence of *Clostridium difficile* colitis, and recovery of the microbiome. The cycle is initiated when the normal colon (A) is depleted of its microbiota using broad spectrum antibiotics (B). Although the microbiota often recovers spontaneously from such treatment, the patient is at risk of *Clostridium difficile* (*C. difficile*) colitis (C and D) in a fashion that is independent of the presence of an appendix. Although *C. difficile* colitis is often effectively treated with metronidazole or vancomycin (E), the microbiome can fail to normalize, leading to recurrent *C. difficile* colitis. This cycle of treatment followed by recurrence is indicated by the red arrows. The presence of a vermiform appendix enhances recovery (A and F) of a normal microbiome following *C. difficile* colitis (green arrow), thus averting the cycle of recurrent *C. difficile* colitis. Colonic microbiota transplants (CMT) are also effective at restoring the normal flora and interrupting the cycle of recurrent *C. difficile* colitis.

small-bowel resection (1.17%) and gastric resection (1.02%)[41], further suggesting that the appendix may be relatively uninvolved in the initial onset of *C. difficile* colitis. In addition, the fact that an intact appendix protects against recurrent (as opposed to the initial onset of) *C. difficile* colitis (see below) argues strongly against this view. However, again, it is not known to what extent the presence or absence of an appendix might affect antibiotic use, the major trigger for *C. difficile* colitis. This factor probably needs to be examined before any firm conclusions can be drawn.

The appendix and recurrent C. difficile colitis

Strong evidence from Im *et al*[42] study indicates that the appendix may play a protective role in recurrent *C. difficile* colitis. Im *et al* found a 2.5-fold increased risk of recurrent *C. difficile* colitis in patients without an appendix compared to those with an appendix. Figure 2 illustrates a possible scenario that potentially explains the connection between the appendix, the initial onset of *C. difficile* colitis, and recurrent *C. difficile* colitis. The central issue revolves around the use of broad spectrum antibiotics which initiate the initial *C. difficile* colitis, and the more limited antibiotic treatments used after the first *C. difficile* infection. The standard of care for recurrent and severe *C. difficile* colitis is oral vancomycin, a treatment that is limited to the lumen of the bowel. Given the position of the appendix out of the main flow of the bowel, it seems likely that it may indeed be effective at protecting the normal flora from oral vancomycin, just as it putatively protects the normal flora from contamination by pathogens in the main fecal stream.

Consistent with the idea that the connection between recurrent *C. difficile* colitis and the appendix involves the bacterial safe-house function of the appendix, recurrent *C. difficile* colitis can be rapidly resolved using fecal microbiome transplants[43-45]. This observation indicates that *C. difficile* colitis is indeed an issue involving a depleted gut

microbiome, thus adding support to the idea that an appendix might help restore the gut microbiome in times of stress. Indeed, proof of a depleted biome in recurrent *C. difficile* colitis patients has been provided by phylogenetic analyses of stool samples in patients with recurrent *C. difficile* colitis: decreased bacterial diversity[46] as well as a deficiency of Firmicutes and Bacteriodetes[47] have been demonstrated in those patients.

Although the function of the appendix as a safehouse for the colonic microbiome explains the clinical observations illustrated in Figure 2, an alternative, although not mutually exclusive, explanation also exists: as noted above, appendectomy probably lowers the immunoreactivity of the gut, and thus may lower the ability of the gut to respond to *C. difficile*. Thus, the loss of the appendix may, hypothetically, reduce the ability of the immune system to mount an immune response to *C. difficile*, which is known to be important in the resolution of the colitis. Thus, a second explanation for the connection between appendectomy and recurrent *C. difficile* colitis shown in Figure 2 may be that the immunosuppressive effect of appendectomy impedes the immune response to *C. difficile*, thus putting the patient at risk for recurrent *C. difficile* colitis. Consistent with this view, Im's data also indicated that increasing age (> 60 years), which is associated with reduced immune function, was also a risk factor for recurrent *C. difficile* colitis[42]. In this view, the lack of a connection between the initial onset of *C. difficile* colitis and appendectomy may be due to the lack of time necessary to mount an immune response that would be dependent on the immune tissue of the appendix.

The appendix and gastrointestinal pathology unrelated to Clostridium difficile

A potentially alarming observation was made in the study by Merchant *et al*[41]: 31 percent (39 out of 121) of their patients which were tested for *C. difficile* colitis but which were found negative for *C. difficile* colitis had a previous appendectomy. This number is very significantly greater than is expected if the presence or absence of an appendix was not related in some way: The probability (binomial test) of observing 38 out of 121 patients with an appendectomy is < 0.0001 given a null hypothesis of 0.18 (a population-wide rate of 18% appendectomy). If this observation is confirmed by additional studies, it would indicate an association between appendectomy and complications which resemble *C. difficile* colitis (and thus induce clinicians to order a test for *C. difficile*), but which are in fact not associated with *C. difficile*. This idea deserves further attention before any firm conclusions can be drawn, but the observations made by Merchant *et al*[41] nevertheless have great potential importance, and certainly raise a sense of urgency for further study of this topic.

The strongest connection between appendectomy and inflammatory diseases unrelated to *C. difficile* colitis of the bowel is provided by the Merchant *et al* study[41]. However, additional indirect evidence for this connection is provided by the effectiveness of colonic microbiota transplants in treating some patients whose disease has resisted other therapeutic options[43,44]. The effectiveness of microbiota transplants in some patients strongly indicates that a loss of the normal microbiome is at the root of the symptoms experienced by these patients. Thus, to the extent that the appendix assists in maintenance of the microbiome, the lack of an appendix may influence the incidence of these idiopathic cases. At the same time, it is recognized that loss of the microbiome by a wide range of modern medical interventions (*e.g.*, sterile birth practices, broad spectrum antibiotics) may circumvent any protective role of the appendix, and direct assessment of the rate of appendectomy in patients with an altered microbiome should be undertaken.

Alternatives to appendectomy

Acute appendicitis is the widely recognized indication for appendectomy, although alternatives involving medical treatment are being considered. Medical treatment alone has the substantial disadvantages that (1) heavy use of antibiotics must be employed, which is not without its own side effects; and (2) recurrence of appendicitis following antibiotic use is possible. A controlled study by Eriksson *et al*[48] compared the outcomes of patients treated with a 10 d antibiotic regimen (cefotaxime and tinidazole in the hospital for two days followed by eight days of oral antibiotics) versus patients who underwent appendectomy. They found that patients on the antibiotic regimen used significantly less morphine, had lower white blood cell counts, and had less pain at follow up. Two surgical patients underwent post-operative antibiotic therapy for complications, and there was an appendicitis recurrence rate of 35% in the antibiotic group. Another study by Styrud *et al*[49] saw an 86% success rate with antibiotics with only a 14% recurrence rate within one year. The complication rate in the surgical group was 14%. These studies suggest that acute non-perforated appendicitis can be treated conservatively with an antibiotic regimen; however, the risk of recurrence should be compared to the risk of surgical complication in the patient.

Antibiotics have also proven effective at delaying appendectomy. Nine sailors who were diagnosed with appendicitis while serving at sea received various antibiotic regimens until the men could be taken to a hospital, and all achieved positive outcomes[50]. A study of 695 children showed that an antibiotic regimen in children allowed the appendectomy to be delayed up to 18 h after admission without an increase in complications[51].

CONCLUSION

It seems highly likely that the appendix, evolved in a time before sewer systems and water treatment facilities existed, is somewhat out of place in post-industrial society. Removal of the appendix and its associated GALT does afford some degree of immune suppression, which can be advantageous in a post-industrial environ-

ment rampant with inflammatory diseases of the bowel. However, removal of the appendix may also impede the ability of the body to replenish helpful bacteria, and/or appendectomy might hinder helpful immune responses, such as those directed at *C. difficile*. Whatever the cause, appendectomy appears to be associated with an increased risk for recurrent *C. difficile* colitis, which is not a minor problem in modern medical practice. Indeed, one study found nosocomial *C. difficile* diarrhea present in 3.4 to 8.4 cases per 1000 hospital admissions[40], and an increase in in-hospital mortality from 2.4% to 13.5%[41]. With this in mind, further studies aimed at biome reconstitution, which are predicted to eliminate the vast majority of appendicitis cases, and thus the need for most appendectomies, are warranted. Further, studies regarding the long term effects of incidental appendectomies should be carefully considered.

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MINIREVIEWS

MicroRNA-21 as a potential colon and rectal cancer biomarker

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Abstract

Colorectal cancer (CRC) is one of the most common malignant diseases worldwide and the prognosis is still poor although much progress has been achieved in recent years. In order to reduce CRC-related deaths, many studies are aimed at identifying novel screening- and prognosis-related biomarkers. MicroRNAs (miRNAs) are a class of 18-27-nucleotide single-stranded RNA molecules that regulate gene expression at the post-transcriptional level. It has been demonstrated that miRNAs regulate a variety of physiological functions, including development, cell differentiation, proliferation, and apoptosis. They play important roles in various physiologic and developmental processes and in the initiation and progression of various human cancers. It has been shown that miRNAs can critically regulate tumor cell gene expression, and evidence suggests that they may function as both oncogenes and tumor suppressor genes. In CRC, miRNAs-21 is one of the most important miRNAs and is rapidly emerging as a novel biomarker in CRC, with good potential as a diagnostic and therapeutic target. In this review, we summarize the latest research findings of the clinicopathological relevance of miRNAs-21 in CRC initiation, development, and progress, highlighting its potential diagnostic, prognostic, and therapeutic application, as well as discussing future prospects.

Key words: Colorectal cancer; MicroRNA; Diagnosis; Treatment; Chemotherapy

Core tip: We summarize the latest study findings about microRNA-21 in colorectal cancer through a systematic review of literature. We recommend microRNA-21 as one of the most important microRNAs, which is rapidly emerging as a novel biomarker, with good potential as a diagnostic and therapeutic target.

Li T, Leong MH, Harms B, Kennedy G, Chen L. MicroRNA-21 as a potential colon and rectal cancer biomarker. *World J Gastroenterol* 2013; 19(34): 5615-5621 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i34/5615.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i34.5615

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and the second leading cause of death in the United States. It was estimated that there were about 142570 new cases diagnosed and 51370 deaths in 2010[1]. Progress in diagnosis and treatment has had a positive effect in improving overall survival, with more patients being diagnosed in the early stage of the disease, but the outcomes of patients diagnosed with advanced stage disease remains quite poor[2]. Long-term survival and better prognosis of patients depend on the stage of the tumor at the time of detection. Fecal occult blood testing and tumor markers (*e.g.*, carcinoembryonic antigen) are used as the primary screening tools, with colonoscopy reserved for patients testing positive. However, they are generally considered to lack the desired convenience, sensitivity and specificity[3]. There are currently no tests or biomark-

ers that precisely predict the presence of early tumors, recurrence, sensitivity to chemotherapy and long-term survival. It is clear that improvements in early detection of primary and recurrent disease are required.

MicroRNAs (miRNAs) are a family of small, non-coding RNAs (19-22 nucleotides) which post-transcriptionally regulate gene expression. In general, miRNAs are transcribed as a group called the pri-miRNA complex, which is cleaved in the nucleus to form the pre-miRNA which is then translocated to the cytoplasm where they undergo final maturation into a functional miRNA[4]. Once in the cytoplasm, the miRNAs regulate gene expression by binding to the 5'-untranslated region of their target mRNA resulting in degradation of the double-stranded mRNA mediated by the Dicer complex. More than 700 miRNAs have thus far been identified in plants, viruses, animals and humans, and this number continues to increase (www.mirbase.org). Studies have shown that about 30% of human genes are regulated by miRNAs[5]. This wide regulation has implications in many important cellular functions including development, differentiation, proliferation, and programmed cell death[6-8]. Given the critical regulatory roles miRNAs serve, it is no surprise that they have been shown to be associated with many cancers[9]. CRC is a complex genetic disease characterized by uncontrolled proliferation, migration, invasion, and failure of apoptotic cell death, due to oncogene activation and tumor suppressor gene defects[10]. Many miRNAs which mediate cell growth and tumor progression have been found to be upregulated in CRC including miR-20, miR-21, miR-17-5p, miR-15b, miR-181b, miR-191 and miR-200c[9,11-14]. While lower levels of mature miRNAs such as miR-34a, miR-126, miR-143, miR-145 and miR-342 are also found, suggesting that they act as tumor suppressor miRNAs[15-18]. This deregulation of various miRNAs has been associated with tumor diagnosis and prognosis indicating that they might be potential biomarker in clinical application[3,19-21]. Multiple studies have identified that miR-21 plays a significant role in cancer biology, diagnostics and prognosis. In this article, we review the literature demonstrating the importance of miR-21 in CRC, summarize the association of miR-21 expression level with CRC diagnosis and prognosis, and discuss the potential therapeutic implications for the future.

MIR21 IN COLORECTAL CANCER

Human miR-21 (hsa-miR-21) was cloned from HeLa cell total RNA and is highly conserved among species including human, rat, mouse, fish and frog[22]. It is located on chromosome 17q23-1 overlapping with the TMEM49 gene, a human homologue of rat vacuole membrane protein-1. MiR-21 encodes a single hairpin and is regulated by its own promoter containing binding sites for AP-1 and PU.1 transcription factors[23]. Experimental data has shown that miR-21 functions in many cell types as an anti-apoptotic and pro-survival factor and plays a significant role in cancer biology and prognosis[24-26]. Asangani *et al*[26] transfected Colo206f cells with miR-21 and found significant suppression of PDCD4 proteins *in vitro*. Resected normal and tumor tissues of 22 CRC patients demonstrated that miR-21 expression has a direct correlation with tumor invasion and metastasis.

Table 1 Current screening methods and guidelines for colorectal cancer

Method	Sensitivity	Interval	Society
Fecal tests			
FOBT		Yearly	USPSTF, ASGE, USMSTF
FIT	65.8%[32,33]	Yearly	
Fecal DNA	50%-60%[34]	Unspecified	USMSTF
Serum markers			
CEA	30%[35]		
CA19-9			
Imaging tests			
DCBE	85%-97%[36]	Every 5 years	USMSTF
CTC	55%-94%[37]	Every 5 years	USMSTF
Optical tests			
FS		Every 5 years Every 10 years	USPSTF, ASGE, USMSTF
FC			USPSTF, ASGE, USMSTF

FOBT: Fecal occult blood test; FIT: Fecal immunochemical based stool tests; CEA: Carcinoembryonic antigen; DCBE: Double-contrast barium enema; CTC: Computed tomography colonography; FS: Flexible sigmoidoscopy; FC: Flexible colonoscopy; USPSTF: United States Preventive Services Task Force; ASGE: American Society for Gastrointestinal Endoscopy; USMSTF: Multi-Society Task Force on Colorectal Cancer.

miR-21 in adenomas

It is clear that the majority of CRCs begin as benign adenomas, and through a series of accumulated genetic events, end up as invasive tumors. However, not all polyps will progress to invasive carcinomas. In fact, it is estimated that up to 20% of benign, subcentimeter adenomas will ultimately regress[27,28]. Therefore, it seems that the key to preventing polyps from progressing to malignant carcinomas is being able to determine which ones have the potential to progress and removing them at the benign stage. Interestingly, increased expression of several miRNAs such as miR-21, miR-31, miR-96, miR-221, miR-191, miR-19a, and miR-135b has been shown to correlate with the presence of adenomas[29,30]. In fact, Yamamichi *et al*[31] analyzed miR-21 expression patterns in different stages of CRC development using *in situ* hybrization, and found higher miR-21 expression in precancerous adenomas but not in non tumorigenic polyps. Furthermore, the frequency and extent of miR-21 expression increased during the transition from precancerous colorectal adenoma to advanced carcinoma. This demonstrates that expression of miR21 in benign colon adenomas may represent an early event in the progression to carcinoma.

Expression of miR-21 as a screening test for colorectal cancer

Current recommendations for CRC screening are found in Table 1[32-37]. Fecal occult blood testing is a widely used test but its low specificity and sensitivity limits its clinical

use, particularly for early detection. Newer screening tests are taking advantage of the presence of stem cells from human exfoliated deciduous teeth cells in the stool and are using various molecular tests to examine these cells for genetic events consistent with malignant changes. Expression levels of miRNAs offer attractive new potential biomarkers as they are uniquely stable and may represent some of the earliest changes in adenomas. Ng *et al*[38] reported high expression levels of miRNAs in colorectal tumors and plasma. Of the panel of 95 miRNAs analyzed by real-time polymerase chain reaction (PCR), five were upregulated in both plasma and tissue. The results were again validated using the plasma of 25 patients with CRC and 20 healthy controls. In these studies, the miRNAs 21, 17-3p, and 92 were elevated in patients with CRC ($P < 0.0005$). The authors further demonstrated that the plasma levels of these markers were significantly reduced after surgery in 10 patients with CRC ($P < 0.05$) suggesting that the high levels specifically indicate the presence of a carcinoma. Kristina *et al* tested the levels of 15 miRNAs in stool and colorectal tissue samples from 15 patients with CRC and five healthy individuals[39]. Although, variability was more pronounced among the stool samples than the tissue samples, the authors concluded that specific miRNA expression profiles could be defined, suggesting that stool is yet another biological material in which miRNAs are preserved and are amenable for early diagnosis of CRC. A stage-independent, sensitive, and specific marker for CRC in plasma or stool would be clinically important, and clearly these promising results support further assessment of miRNAs as potential biomarkers both in adenoma and extracellular fluids.

miR-21 expression levels and prognosis

The prognosis of patients with CRC is associated with tumor stage and phenotypic characteristics of resected cancer specimens such as tumor grade, positive lymph nodes, and angiolymphatic invasion[40]. Unfortunately, recently identified genomic and proteomic biomarkers, tumor cell mutations, and microsatellite instability cannot be recommended for routine clinical use because of insufficiently available data[41]. However, markers of prognosis are needed to help stratify patients into high risk thereby identifying patients who are likely to benefit from further therapy. Many studies on tumor biomarkers have been undertaken[42]. However, no study has identified a new marker that has been validated in clinical trials. The miRNAs represent particularly attractive markers as they seem to be micromanagers of cellular gene expression and may represent the earliest events responsible for carcinogenesis. In fact, studies have shown that the expression levels of different miRNAs, such as miR-21, miR-320, miR-498, miR-106a and miR-200c, correlate with disease-free and overall survival[43].

miR-21 may be a particularly attractive target as it has been shown to regulate the expression of many genes thought to be important in carcinogenesis. Target validation studies on putative miR-21 targets in breast cancer samples and CRC cells have demonstrated a link between miR-21 expression levels and the p53 tumor suppressor, and also demonstrated that the tumor suppressors PDCD4 and maspin are targets of miR-21[44]. Consistent with the importance in the process of carcinogenesis, Staby *et al*[45] demonstrated that higher miR-21 expression levels were correlated with advanced cancer stages, worse outcome, poor response to therapy, and shorter disease-free survival. Additionally, they found that miR-21 levels were positively correlated with cancer stage, lymph node involvement, and development of distant metastasis.

The most comprehensive analysis of miRNA expression in CRC performed to date tested two cohorts of 197 colon cancer patients by utilizing microarrays containing 389 miRNAs probes[46]. This analysis revealed 37 miRNAs which were differentially expressed in stage Ⅱ colonic adenocarcinoma compared with adjacent normal tissue using a test set and two validation cohorts. In one of the cohorts, miR-20a, miR-21, miR-106a, miR-181b, and miR-203 were found to be overexpressed in tumor tissues with high tumor to normal ratios, as well as being associated with poor overall survival. However, the prognostic relevance could be confirmed in the validation set for only one of the candidates, miR-21, and the clinical and biological implications of differential expression of the remaining miRNAs were unclear. Similar conclusions were drawn from a study of 29 tumor samples, in which miR-21 expression was associated with poor survival and therapeutic outcome in stage Ⅱ and Ⅲ CRC[46]. Nielsen *et al*[47] reported the expression of miRNA-21 in 130 colon and 67 rectal stage Ⅱ cancer specimens using high-affinity locked nucleic acid (LNA) probes. High levels of miR-21 correlated with shorter disease-free survival (hazard ratio: 1.28; 95% confidence interval: 1.06-1.55; $P = 0.004$) in the stage Ⅱ colon cancer patient group, whereas no significant correlation with disease-free survival was observed in the stage Ⅱ rectal cancer group.

miR-21 expression and implications for treatment

miR-21 and response to chemotherapy: The current treatment for CRC involves a multidisciplinary approach including surgery supplemented with chemotherapy and radiation therapy in certain instances. In general, patients with node-positive disease benefit from chemotherapy. However, there may be a subgroup of patients with node-positive disease who are at low risk of recurrence, as nearly 40% of patients randomized to a no-treatment arm in chemotherapy trials did not develop a recurrence[48]. In addition, it is clear that some patients with node-negative disease who have advanced T-stage tumors are at high risk of developing recurrences[49]. A test that would allow for the accurate stratification of patients with stage Ⅱ and Ⅲ disease into low and high risk would be very clinically useful. Recently, the role of miRNAs in predicting the response to 5-fluorouracil (5-FU)-based chemotherapy in CRC treatment has been explored. A significant focus has been placed on the value of miR-21 expression levels and their abilities to predict both response to and need for chemotherapy[50].

Rossi *et al*[51] utilized two subclones from the human

Table 2 MicroRNA-21 expression in colorectal cancer

Ref.	Regulation	Biological material tested	Detection method	Clinical relevance	Comment
Tumor development					
Fassan *et al*[60]	Up	300 polypoid lesions of the colon mucosa	RT-PCR ISH	Significant miR-21 upregulation in preneoplastic/neoplastic samples	High miR-21 expression is consistent with PDCD4 downregulation
Yantiss *et al*[61]	Up	24 patients < 40 years 45 patients ⩾ 40 years	RT-PCR	Significantly higher expression	
Tumor diagnosis					
Link *et al*[62]	Up	Stool samples	RT-PCR	Higher expression in patients with adenomas and CRCs	May be an excellent candidate of a noninvasive screening test for colorectal neoplasms
Tumor prognosis					
Chang *et al*[63]	Up	48 colorectal tumors, 61 normal tissues , 7 polyps	RT-PCR	Disease recurrence	miR-21 post-transcriptionally modulates PDCD4 *via* mRNA degradation
Nielsen *et al*[47]	Up	130 stage Ⅱ colon and 67 stage Ⅱ rectal cancer specimens	ISH	Shorter disease-free survival in colon cancer, but not in rectal cancer	
Kulda *et al*[64]	Up	46 paired tissue samples 30 tissue samples with live metastasis	RT-PCR	Disease-free interval	
Schetter *et al*[46]	Up	196 paired tissues	RT-PCR	Association with cancer-specific mortality, including stage Ⅱ patients alone	miR-21 expression are independent predictors of colon cancer prognosis and may provide a clinically useful tool to identify high-risk patients
Schetter *et al*[46]	Up	US cohort: 84 patients; Hong Kong cohort: 113 patients	MicroRNA microarray, RT-PCR	Poor survival and poor therapeutic outcome	

RT-PCR: Reverse transcription-polymerase chain reaction; ISH: *In situ* hybridization; PDCD4: Programmed cell death protein 4.

CRC cell lines HT29 and HCT116 to investigate the effect of 5-FU on miRNA expression and also to determine patterns of expression that correlated with response to therapy. Quantitative real-time PCR revealed that 5-FU upregulated 19 and downregulated three miRNAs. While some changes in miRNA expression were consistent with the antitumor effects of the drug, others were not, such as upregulation of miR-21 and the polycistronic miR-17-92 cluster (which include miR-19a and miR-20). In fact, a number of miRNAs that are already overexpressed in neoplastic tissues, including miR-21, have been shown to be upregulated in colon cancer cell lines treated with 5-FU[51]. Tomimaru *et al*[52] found that hepatocellular carcinoma cells transfected with pre-miR-21 were significantly resistant to 5-FU, while the 5-FU sensitivity of transfected anti-miR-21 was weakened by transfection with siRNAs of the target molecules, PTEN and PDCD4. This finding may be a cell-specific defense mechanism to survive 5-FU treatment. Svoboda *et al*[53] found significant changes in miRNA expression in 35 patients with rectal carcinoma undergoing preoperative capecitabine chemoradiation therapy. Tumor biopsies were taken before starting therapy and after 2 wk of therapy. The extent of the tumor response to the therapy was investigated microscopically by an experienced pathologist according to Mandard's tumor regression criteria. In addition, the levels of miRNAs were evaluated using real-time PCR. The authors found dramatic changes in the expression levels of many miRNAs including miR-21, miR-10a, miR-145, miR-212, miR-339, miR-361. However, only two miRNAs, miR-125b and miR-137, were found to be significantly increased after 2 wk of therapy and these miRNA expression levels had a positive correlation with a poorer tumor regression response[53]. These types of studies highlight the potential for using miRNA expression profiles to predict the response to chemotherapy. However, verification of the targets in adequately designed clinical panels is the important next step that has yet to be taken.

miR-21 maybe a potential therapeutic target in colorectal cancer treatment: miRNAs are important regulators of gene expression and may present potentially interesting therapeutic targets in cancer. The synthesis, maturation and activity of miRNAs can be manipulated with various oligonucleotides that encode the sequences complementary to mature miRNAs. By influencing particular miRNAs, a cascade of pathways could be modified to inhibit tumor growth.

Wong *et al*[54] reported the application of 20-*O*-methyl- and/or DNA/LNA-mixed oligonucleotides to specifically inhibit miR-21 in cultured glioblastoma and breast cancer cells suppressed cell growth *in vitro* in association with increased caspase-mediated apoptosis[55]. Suppression of miR-21 also significantly reduced invasion and lung metastasis in MDA-MB-231 metastatic breast cancer[56]. Although there are at present no clinical reports describing therapy targeting miR-21 in CRC treatment,

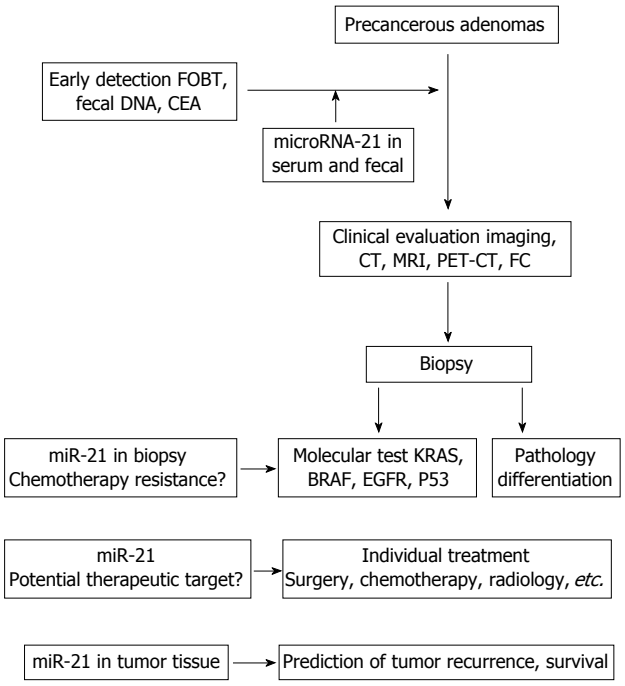

Figure 1 miR-21 as a potential biomarker in colon and rectal cancer. CT: Computed tomography; MRI: Magnetic resonance imaging; PET-CT: Positron emission tomography-computed tomography; FC: Fiber colonscopy; FOBT: Fecal occult blood test; CEA: Carcinoembryonic antigen.

most seem to have optimistic views on the future utility of miR-21 as therapeutic targets but further studies are clearly needed[57-59]. Expression of microRNA-21 and the clinical relevance in CRC are summarized in Table 2.

FUTURE PERSPECTIVES

The role of miRNAs in CRC presents potentially exciting new opportunities for future investigations to determine the use of miRNAs as potential biomarkers for prognosis at the time of diagnosis as well as to determine their ability to predict the response to chemotherapy. In addition, the potential for miRNAs to serve as targets for new chemotherapeutic treatments has yet to be realized. Among the many miRNAs that have been associated with clinical outcomes, miR-21 has been consistently shown to be dysregulated in CRC. Many of the early studies relating miR-21 to CRC have been performed *in vitro* on established cell lines. In addition, studies using human tumor tissue have been performed in a retrospective fashion, which limits the conclusions because of inherent study bias. If these micromanagers of cell processes are to be useful tools in the diagnosis or treatment of colon cancer, we will have to study them in well-designed prospective trials. It remains to be discovered if these types of molecular expression profiles will be used in clinical practice (Figure 1).

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ORIGINAL ARTICLE

Doxorubicin-eluting bead *vs* conventional transcatheter arterial chemoembolization for hepatocellular carcinoma before liver transplantation

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Abstract

AIM: To assess the possible effect of two different types of preoperative transcatheter arterial chemoembolization (TACE) on recurrence-free survival after liver transplantation (LT) in patients with hepatocellular carcinoma (HCC) and to analyze the effects of TACE on tumor histology.

METHODS: We retrospectively analyzed the histological features of 130 HCC nodules in 63 native livers removed at transplantation. Patients who received any other type of treatment such as radiofrequency tumor ablation, percutaneous ethanol ablation or who were not treated at all were excluded. All patients in the present study were within the Milan Criteria at the last imaging findings before transplantation. Doxorubicin-eluting bead TACE (DEB-TACE) was performed in 22 patients (38 nodules), and conventional TACE (c-TACE) in 16 (25 nodules). Patients' and tumors' characteristics were retrospectively reviewed. We performed a per-nodule analysis of the explanted livers to establish the mean percentage of necrosis of any nodule treated by TACE (conventional or DEB) and a per-patient analysis to establish the percentage of necrosis in the cumulative tumor area, including 21 nodules not reached by TACE. Inflammatory and fibrotic changes in the tissue surrounding the tumor nodule were analyzed and categorized as poor/absent, moderate and enhanced reaction. Uni- and multivariate analysis of risk factors for HCC-recurrence were performed.

RESULTS: The number and diameter of the nodules, the time spent on the waiting list and the number of treatments were similar in the two groups. A trend towards higher appropriate response rates (necrosis ⩾

90%) was observed in the DEB-TACE group (44.7% *vs* 32.0%, $P = 0.2834$). The mean percentage of necrosis in the cumulative tumor area was 58.8% ± 36.6% in the DEB-TACE group and 50.2% ± 38.1% in the c-TACE group ($P = 0.4856$). Fibrotic and inflammatory reactions surrounding the tumor nodule were markedly more common in the DEB-TACE group ($P < 0.0001$, for both the parameters). The three-year recurrence-free survival was higher in DEB-TACE-treated patients than in conventionally treated patients (87.4% *vs* 61.5%, $P = 0.0493$). Other factors affecting recurrence-free survival included viable tumor beyond Milan Criteria on histopathological examination, the percentage of necrosis on CTA ⩽ 50% and a pre-transplant serum α-fetoprotein level greater than 70 ng/mL. On multivariate analysis, the lack of treatment with DEB-TACE, high levels of α-fetoprotein and viable tumor beyond Milan Criteria at histology examination were identified as independent predictors of tumor recurrence.

CONCLUSION: DEB-TACE can effectively promote tumor necrosis and improves recurrence-free survival after LT in HCC.

Key words: Liver transplantation; Hepatocellular carcinoma; Transcatheter arterial chemoembolization; Doxorubicin-eluting bead; Tumor histology; Recurrence-free survival; Locoregional therapies

Core tip: The manuscript reports the experience with a newer technique of transcatheter arterial chemoembolization (TACE) that uses doxorubicin-eluting beads (DEB) for the treatment of hepatocellular carcinoma in liver transplant candidates. The results of DEB-TACE were compared to those of conventional TACE, and remarkably, a significantly higher recurrence-free survival after liver transplantation was observed in patients who were treated with DEB-TACE. The histological pattern observed in the area surrounding the tumor nodules of DEB-TACE patients was characterized by an intense inflammatory and fibrotic reaction, which could play a role in limiting tumor spread during waiting list time.

Nicolini D, Svegliati-Baroni G, Candelari R, Mincarelli C, Mandolesi A, Bearzi I, Mocchegiani F, Vecchi A, Montalti R, Benedetti A, Risaliti A, Vivarelli M. Doxorubicin-eluting bead *vs* conventional transcatheter arterial chemoembolization for hepatocellular carcinoma before liver transplantation. *World J Gastroenterol* 2013; 19(34): 5622-5632 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i34/5622.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i34.5622

INTRODUCTION

Hepatocellular carcinoma (HCC) accounts for approximately 5% of all cancers, with more than 500000 new cases diagnosed each year[1]. The link between liver cirrhosis and HCC is well known; more than 90% of HCCs develop in cirrhotic livers, and 3%-8% of cirrhotic patients are diagnosed as HCC carriers each year[2]. Ideally, liver transplantation (LT) is the best treatment option for HCC, as it removes both the tumor and the underlying chronic condition[3].

Milan Criteria (MC) of LT candidates with HCC, based on the number and size of the tumor nodules, has led to 5-year survival rates well above 70% and recurrence rates below 15%[4]. Currently, more than 30% of LT recipients in the United States are HCC carriers[5].

In an intent-to-treat purpose, one of the major limitations of LT in HCC carriers is the time spent on the waiting list; the risk of tumor progression increases with time, resulting in a cumulative probability of dropout from the waiting list of 7.2% for a 6-mo waiting time, which rises to 37.8% and 55.1% for 12 and 18 mo of waiting time, respectively[6].

To attempt to cure to a larger number of HCC carriers, two strategies have been outlined. The first is to downstage those tumors that exceed the Milan Criteria at the time of the first observation, thereby allowing transplantation. The second strategy is to delay the tumor growth using locoregional treatments while the patient is on the waiting list to reduce the dropout rate. The response to locoregional treatment is related to patient prognosis and seems to denote favorable tumor biology[7-10].

Transcatheter arterial chemoembolization (TACE) is the most frequently used treatment of HCC in LT candidates[11]. TACE is usually performed by administering a mixture of epirubicin and Lipiodol to concentrate the drug within the tumor. This is followed by a gelatin sponge (conventional TACE, c-TACE) to obtain occlusion of the feeding arteries of the tumor, with the aim of producing infarction and necrosis of the tumor tissue. Recently, a novel doxorubicin-eluting bead (DEB) has been developed to bind, deliver and elute chemotherapeutic drugs in the tumor area during TACE. Pre-clinical and clinical studies have demonstrated that DEB-TACE produces a higher drug concentration within the tumor than c-TACE while maintaining a lower systemic concentration[12-14].

The assessment of the tumor response after TACE remains a critical issue; the Response Evaluation Criteria in Solid Tumors (RECIST), based on the sum of the largest diameter of target lesions on computed tomography (CT) or magnetic resonance (MR) before and during treatment, can be misleading when assessing the treatment-related tumor necrosis, which is not necessarily associated with a reduction in tumor diameter[15,16]. In 2001, a panel of experts concluded that an estimation of the reduction of viable tumor (recognized as the non-enhancing areas on a CT scan) should be considered the optimal method to assess local response (modified-RECIST)[17].

However, CT findings often underestimate the residual tumor extent, which can be accurately determined only at histology[18]; in this regard, LT represents a unique set-

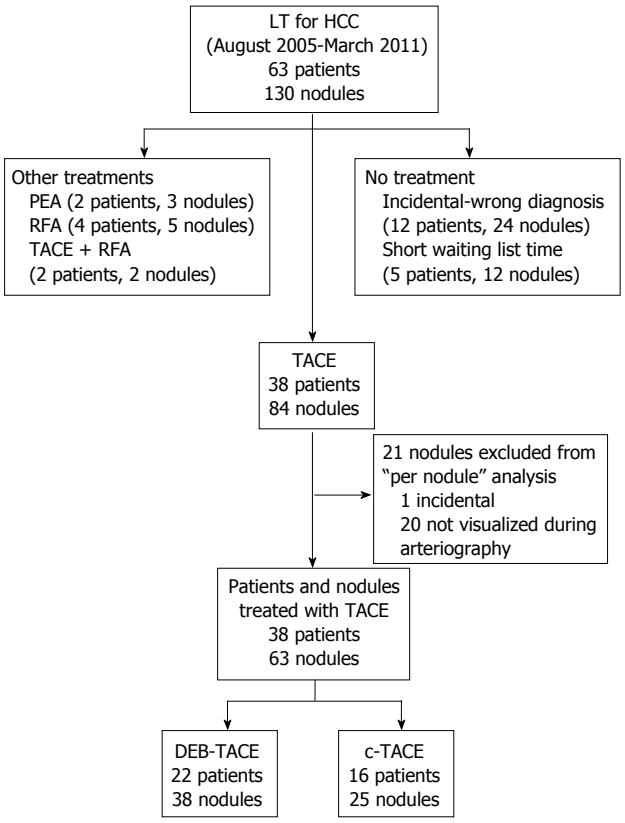

Figure 1 Flow chart of the patients included in the study. TACE: Transcatheter arterial chemoembolization; DEB: Doxorubicin-eluting bead; HCC: Hepatocellular carcinoma; LT: Liver transplantation; PEA: Percutaneous ethanol ablation; RFA: Radiofrequency tumor ablation; c-TACE: Conventional TACE.

ting to correctly assess tumor necrosis induced by TACE, as the whole native liver becomes available for histological examination.

Although excellent tumor necrosis rates induced by TACE are reported in LT recipients[19], the impact of TACE on recurrence-free survival remains to be established[20-22].

The aim of this study was to compare DEB-TACE with c-TACE by assessing the histological features of the tumor nodules in native livers removed at transplantation, focusing on the degree of necrosis, as well as to assess the recurrence-free survival of HCC recipients after LT.

MATERIALS AND METHODS

From August 2005 to March 2011, 63 liver transplants were performed in patients with HCC on cirrhosis in our center. We retrospectively analyzed the histological features of 130 HCC nodules in 63 native livers removed at transplantation (2.06 nodules per patient). Patients who received any other type of treatment, including radiofrequency tumor ablation (RFA) or percutaneous ethanol ablation (PEA), or those who were not treated at all were excluded from the present analysis, as described in Figure 1. Thirty-eight patients who received one or more TACEs as the only neoadjuvant therapy before LT represent the study population.

The policy of our center for TACE is to downstage those tumors that are initially beyond the MC. In the present study, only those patients who were successfully downstaged within the MC and therefore underwent LT are considered; TACE was also performed in those patients who fulfilled the MC and entered the waiting list with an expected waiting time longer than 2 mo. Based on the imaging findings, all the patients in the present study were within the Milan Criteria at the time of transplantation.

As the initial endpoint of our study was to confirm the safety of DEB-TACE and to assess its efficacy in achieving tumor necrosis in comparison with c-TACE, we performed a per nodule analysis of the explanted livers to establish the mean percentage of necrosis of any nodule treated by TACE (conventional or DEB). Twenty-one nodules were not reached by the treatment due to the failure to visualize the tumor's feeding arteries during arteriography (20 cases) or failure to visualize the tumor itself in the pre-LT imaging (1 case); these 21 nodules were not included in the "per nodule" analysis to assess the mean percentage of necrosis produced by TACE. However, these nodules were taken into account in the "per patient analysis" and in the "survival analysis" to precisely quantify the neoplastic burden of each patient, which could influence the prognosis.

Demographics (age, sex), etiology of cirrhosis, the Child-Pugh and the Model for End Stage Liver Disease (MELD score), radiological and pathological tumor classification according to MC, laboratory tests, imaging studies and pathology reports were recorded for each patient. Factors related to tumor biology, such as serum alpha-fetoprotein, microvascular invasion (MVI) and grading, that play a key role in determining tumor recurrence[23-26] were compared between the 2 groups. The waiting time for LT and the interval between the last TACE and LT were also calculated. Computed tomography scans were performed one month after TACE and every 3 mo thereafter; candidates who were initially beyond MC at imaging were reassessed by two interventional radiologist according to the European Association for the Study of the Liver guidelines[17] to define the amount of tumor necrosis after TACE. When radiologic findings demonstrated viable tumor beyond MC, chemoembolization was repeated.

In addition to the type of TACE, the impact of the following risk factors on recurrence-free survival was also assessed: adherence to MC at pathology (considering only the viable portion of each nodule), tumor grading (G3-G4), the presence of MVI, the presence of multiple nodules at pathology, a percent necrosis in the cumulative tumor area (CTA) less than or equal to 50%, high levels of α-fetoprotein (> 70 ng/mL) and the need to repeat TACE before LT.

Transcatheter arterial chemoembolization

All patients underwent baseline celiac and superior mesenteric arteriography *via* a femoral artery approach. Prior to embolization, liver vascular anatomy was identified

to check the patency of the portal vein and visualize the arterial feeders of the tumor(s). The procedure was defined as "superselective" when the tip of a highly flexible coaxial microcatheter (2.7 Fr; Progreat; Terumo) was successfully placed in the branches supplying the tumor. When nodules were fed by multiple tiny arteries or when multinodular disease was present, TACE was performed with segmental or lobar (only for c-TACE group) catheterization (non-superselective TACE). Conventional TACE was performed by administering a mixture of 50 mg of epirubicin (Pfizer, New York, NY, United States) in an emulsion with lipiodol (Guebert, Aulnay-sousBois, France), followed by embolization with gelatin sponge particles (SPONGOSTAN; Johnson and Johnson, Gargrave, United Kingdom). DC beads (Biocompatibles, Farnham, Surrey, United Kingdom) became available at our institution beginning in June 2007; thereafter, patients were randomly assigned to one of the two techniques. The caliber of beads was chosen based on the type of catheterization, the vascularity of the lesion, and the tumor diameter. DEB-TACE was performed using 100- to 300-μm beads for single lesions < 50 mm without arteriovenous shunts, whereas in larger tumors, multiloculated lesions or suspected satellites, one vial of 100- to 300-μm beads and one vial of 300- to 500-μm beads were injected. DC beads were impregnated with 75 mg of doxorubicin in each vial to a maximum of 150 mg of doxorubicin loaded in two vials of DC beads (4 mL total).

Histopathology

After LT, a dedicated liver pathologist performed the analysis of all the explanted livers, which were serially cut into sections of approximately 0.5 cm in thickness. The presence of cirrhosis was confirmed in all cases. Every lesion suspected to be HCC was completely paraffin-embedded, and multiple histological sections were made. Tumor grade according to the Edmonson and Steiner[27] classification and the presence of MVI were also assessed, except when complete necrosis of the tumor was achieved. The necrosis rate of each nodule was expressed as the percentage of necrotic tissue within the whole area of the nodule; necrosis was categorized as complete (100%), appropriate (90% or greater), partial (between 51% and 89%), or inadequate (50% or lower) as described previously[28,29]. The sum of the tumor diameters, CTA, and the cumulative necrotic and viable areas (including not-treated nodules) were measured in each patient to calculate the percentage of tumor necrosis within the cumulative tumor area (% necrosis on CTA). Adherence to MC was assessed during the pathological examination using only the viable portion of each nodule. Inflammatory and fibrotic changes in the tissue surrounding the tumor nodule were analyzed and categorized as poor/absent, moderate or enhanced reaction. The localization of microspheres with respect to the 38 HCC nodules treated with DEB-TACE was assessed and defined as intratumoral (exclusively within the tumor capsule), peritumoral (outside the nodule but within 5 mm of the tumor capsule), intra- and peritumoral or intratumoral (microspheres found in the cirrhotic parenchyma beyond 5 mm from the tumor capsule).

Statistical analysis

Continuous variables were reported as the mean and standard deviation or as median and range and were compared using Student's *t* test or Mann-Whitney *U* test when appropriate. Categorical variables were reported as numbers and percentages and compared using Fisher's exact test. Recurrence-free survival was calculated from the day of surgery to the first follow-up visit at which tumor recurrence was diagnosed or, in patients without recurrence, to the most recent follow-up visit. Follow-up of those patients who died without evidence of recurrence was censored at the time of death. The impact of each individual variable in determining HCC recurrence-free survival was assessed using the Kaplan-Meier method and compared using the log-rank test. Continuous variables, including pre-LT serum alpha-fetoprotein levels and the percentage of necrosis on CTA, were dichotomized; cutoff values were defined according to receiver operating characteristic curve analysis[30]. To identify factors independently related to HCC recurrence, the multivariate Cox proportional-hazard regression analysis was applied, taking in account only the variables that proved significant in the univariate analysis. A two-sided *P* value of less than 0.05 was considered statistically significant in all cases.

RESULTS

Twenty-two patients underwent DEB-TACE, and 16 underwent c-TACE. Age at LT, gender, etiology and severity of cirrhosis were comparable in the two treatment arms (Table 1).

No major complications were observed after TACE. The post-embolization syndrome (transient fever, abdominal pain, nausea) was the most common complication following chemoembolization in both groups; all side effects were successfully treated with medical therapy. The median time spent on the waiting list was similar in the two groups (3.3 mo in the c-TACE, *vs* 2.9 mo in the DEB-TACE group, respectively, $P = 0.5844$).

DEB-TACE and c-TACE were repeated in 12 (54.5%) and 8 (50%) patients, respectively; the maximum number of treatments per patient was 3. The post-LT mean follow-up time was 34.9 ± 19.0 and 46.8 ± 25.6 mo for the DEB-TACE and c-TACE groups, respectively ($P = 0.1065$). No patients were lost at follow-up.

Nodule analysis according to the type of TACE

The size and focality of the tumors were comparable in the two groups at explant examination. The mean tumor necrosis was 55.7% ± 41.9% and 52.2% ± 40.9% in DEB- and c-TACE groups, respectively ($P = 0.7420$). A trend towards a higher probability of an appropriate response was observed in the DEB-TACE group (17/38,

Table 1 Baseline characteristics of the study population *n* (%)

Variable	All treated patients (*n* = 38)	Type of TACE		*P* value
		DEB-TACE (*n* = 22)	c-TACE (*n* = 16)	
Age at LT (yr)	56.5 ± 6.5	57.2 ± 6.5	55.6 ± 6.5	0.4545
Male gender	34 (89.5)	19 (86.4)	15 (93.7)	0.6245
MELD score	10 (6-27)	9 (6-27)	10 (7-16)	0.4688
Etiology of cirrhosis				0.1834
HCV-related	22 (57.9)	10 (45.5)	12 (75.0)	
HBV-related	11 (28.9)	8 (36.3)	3 (18.7)	
Non-viral	5 (13.2)	4 (18.2)	1 (6.3)	
Waiting list time (mo)	3.1 (0.1-26.7)	2.9 (0.1-24.3)	3.3 (0.6-26.7)	0.5844
Interval between last TACE and LT (mo)	3.6 (0.1-15.9)	2.3 (0.2-13.8)	5.5 (0.9-15.9)	0.0625
Repeated TACE	20 (52.6)	12 (54.5)	8 (50.0)	0.7817
Adherence to MC at imaging before TACE				0.3243
Within MC	21 (55.3)	14 (63.6)	7 (43.7)	
Beyond MC	17 (44.7)	8 (36.4)	9 (56.3)	
BCLC stage before TACE				0.3243
A	21 (55.3)	14 (63.6)	7 (43.7)	
B	17 (44.7)	8 (36.4)	9 (56.3)	
Number of nodules before TACE	2 (1-5)	2 (1-5)	2 (1-5)	0.8708
Nodule number class before TACE				0.2222
1 nodule	16 (42.1)	8 (36.4)	8 (50.0)	
1 < nodules < 4	13 (34.2)	10 (45.4)	3 (18.8)	
Nodules ⩾ 4	9 (23.7)	4 (18.2)	5 (31.2)	
[1]Serum α-fetoprotein > 70 ng/mL	8/33 (24.2)	3/18 (16.7)	5/15 (33.3)	0.4811
Post-LT follow-up (mo)	39.9 ± 22.5	34.9 ± 19.0	46.8 ± 25.6	0.1065

Continuous variables are reported as the mean and standard deviation or median and range and compared using Student's *t* test or the Mann-Whitney *U* test as appropriate. Categorical variables are reported as numbers and percentages. [1]Value of serum α-fetoprotein was not available for 5 patients. TACE: Transcatheter arterial chemoembolization; DEB: Doxorubicin-eluting bead; MC: Milan Criteria; LT: Liver transplantation; HCV: Hepatitis C virus; HBV: Hepatitis B virus; MELD: Model for End Stage Liver Disease; c-TACE: Conventional TACE; BCLC: Barcelona Clinic Liver Cancer.

44.7% of nodules) in comparison with the c-TACE group (8/25, 32.0% of nodules), although this difference was not statistically significant (P = 0.2834). No difference in necrosis was found comparing the superselective procedures performed with DEB-TACE or c-TACE (76.2% ± 33.8% *vs* 69.1% ± 36.5%, P = 0.5803). However, independent of the type of TACE (DEB or conventional), superselective procedures resulted in a higher percentage of necrosis than did non-superselective procedures (73.9% ± 34.3% *vs* 31.3% ± 37.0%, P = 0.0018) (Table 2).

Microscopically, tumor necrosis was mixed, colliquative and coagulative in all cases. In the DEB-TACE group only, a "foreign body reaction" with granulomatosis giant cells was observed in the tissue surrounding the tumor in 20 out of 22 patients (90.1%). As described in Figure 2, the nonspecific acute inflammatory infiltrate, containing foamy histiocytes and lymphocytes, was more enhanced at the tumor periphery of DEB-TACE-treated nodules in comparison with the c-TACE-treated ones. In most cases, DEB-TACE-treated nodules were surrounded by thick walls of tissue made of degenerated collagen fibers, inflamed granulation tissue, and hyalinization. The peritumor fibrous tissue of the nodules treated with c-TACE was thinner and apparently less affected by the secondary changes induced by DEB-TACE. These features were not dependent on the time between last treatment and LT; patients with nodules surrounded by an enhanced or mild fibrotic reaction were transplanted 4.2 ± 4.0 mo after TACE, whereas patients with poor-absent fibrosis were transplanted 6.0 ± 4.1 mo after TACE (P = 0.2024).

The distribution of microspheres with respect to the 38 nodules treated by DEB-TACE was intratumoral (11/38, 28.9%), intra- and peritumoral (14/38, 36.8%), peritumoral (10/38, 26.3%) and intratumoral (3/38, 7.9%). Nodules' necrosis differed with respect to the distribution of beads (85.9%, 57.0%, 34.5% and 10.0% for intratumoral, intra- and peritumoral, peritumoral and intratumoral distribution, respectively, P = 0.0041).

Patient analysis according to the type of TACE

Ten of 17 patients were successfully downstaged within the MC; the effectiveness of DEB-TACE and c-TACE was similar to this regard. Seven out of 17 patients who were beyond MC at the imaging performed before TACE (8 patients in DEB-TACE and 9 in c-TACE group) remained outside the MC at pathology; the failure to accurately stage the tumor during the imaging performed before the LT was related to the misdiagnosis of complete necrosis (Table 3).

The number of nodules that were not reached by TACE (21/84, 25%) was similar in the 2 groups (13/51, 25.4% in DEB-TACE and 8/33, 24.2% in c-TACE group, P = 0.8934). The mean diameter of the missed nodules was 1.1 ± 0.5 cm, and 10 out of these 21 nodules (47.6%) were equal or inferior to 1 centimeter in size. The number of HCC nodules, the sum of the tumor diameters and the CTA (also including untreated nodules)

Table 2 Analysis of the treated nodules according to the type of transcatheter arterial chemoembolization (per nodule analysis) *n* (%)

Variable	All treated nodules 63 in 38 patients	Type of TACE		*P* value
		DEB-TACE 38 in 22 patients	c-TACE 25 in 16 patients	
Degree of necrosis	54.3% ± 41.2%	55.7% ± 41.9%	52.2% ± 40.9%	0.7420
Complete necrosis (100%)	21 (33.3)	14 (36.8)	7 (28.0)	0.5877
Histological response				0.2834
Appropriate (necrosis ⩾ 90%)	25 (39.7)	17 (44.7)	8 (32.0)	
Partial (50% < necrosis < 90%)	9 (14.3)	3 (7.9)	6 (24.0)	
Inadequate (necrosis ⩽ 50%)	29 (46.0)	18 (47.4)	11 (44.0)	
Diameters of nodules (cm)	2 (0.7-10)	1.8 (0.7-4.5)	2.2 (1-10)	0.1752
Number of nodules				0.2492
Single	17 (27.0)	8 (21.1)	9 (36.0)	
Degree of necrosis	63.1% ± 37.8%	69.7% ± 34.8%	57.2% ± 41.5%	0.5144
Multiple	46 (73.0)	30 (78.9)	16 (64.0)	
Degree of necrosis	51.1% ± 42.3%	52.0% ± 43.4%	49.4% ± 41.7%	0.8454
Modality of TACE				0.3015
Superselective	34 (54.0)	23 (60.5)	11 (44.0)	
Degree of necrosis	73.9% ± 34.3%	76.2% ± 33.8%	69.1% ± 36.5%	0.5803
Non-superselective	29 (46.0)	15 (39.5%)	14 (56.0)	
Degree of necrosis	31.3% ± 37.0%	24.3% ± 33.3%	38.9% ± 40.5%	0.2970

Analysis performed considering nodules reached by transarterial treatment (targeted lesions). Continuous variables are reported as median and range or mean and standard deviation and compared using the Student's *t* test or Mann-Whitney *U* test as appropriate. TACE: Transcatheter arterial chemoembolization; DEB: Doxorubicin-eluting bead; c-TACE: Conventional TACE.

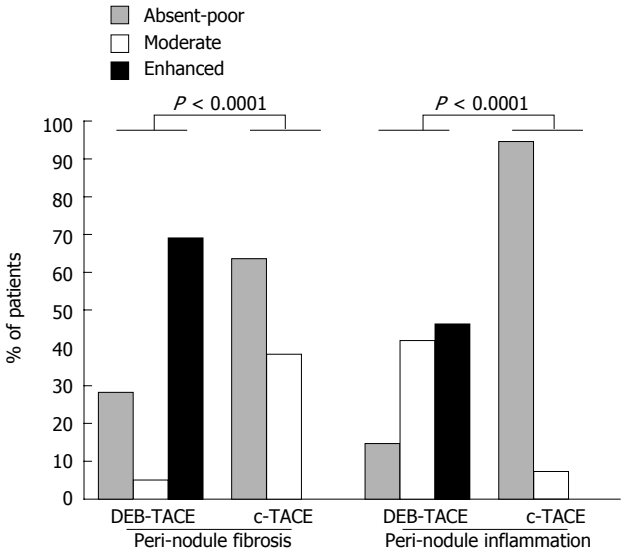

Figure 2 Inflammatory and fibrotic changes in the tissue surrounding the tumor nodules. TACE: Transcatheter arterial chemoembolization; DEB: Doxorubicin-eluting bead.

were similar in the two groups. The mean percentage of necrosis on CTA was 58.8% and 50.2% in the DEB- and c-TACE group, respectively ($P = 0.4856$); no difference in terms of response to treatment in various subcategories was observed. Risk factors for recurrence, such as a pre-transplant serum α-fetoprotein greater than 70 ng/mL (Table 1), tumor grading and microvascular invasion were similarly distributed in the two treatment arms.

Recurrence and survival after LT

Overall 3-year survival after LT was 73.9% and 58.7% in the DEB-TACE and c-TACE groups, respectively ($P = 0.7511$). Seven (18.4%) patients experienced tumor recurrence after LT; the main site of recurrence was the liver (5 patients), the spinal cord and the liver concurrently (1 patient) and the adrenal gland (1 patient). The mean time to recurrence was 17.0 ± 5.5 mo; three out of seven patients were alive with recurrence at the time of publication.

The three-year recurrence-free survival was significantly higher in patients who were treated preoperatively with DEB-TACE than with c-TACE (87.4% *vs* 61.5%, $P = 0.0493$, Figure 3A).

Other factors affecting recurrence-free survival included Milan Criteria unfulfilled at pathology, percentage of necrosis on CTA lower than 50% and pre-transplant serum α-fetoprotein levels greater than 70 ng/mL (Figure 3B-D). On multivariate analysis, a lack of treatment with DEB-TACE, serum α-fetoprotein levels exceeding 70 ng/mL and Milan Criteria unfulfilled at pathology were independent predictors of tumor recurrence (Table 4).

DISCUSSION

Among patients with HCC awaiting LT, TACE is the most commonly used neo-adjuvant therapy[11]. Although TACE can successfully downstage 24% to 63% of HCCs, pre-LT treatment is not clearly associated with any survival benefit[22,31-33]; in a large multicenter study, the 5-year recurrence-free survival was 67% in patients treated with TACE prior to LT and 64% in those not treated[20].

Unlike conventional TACE, which is the most commonly used technique, DEB-TACE is based on calibrated microspheres made of non-degradable polymers that produce permanent vascular embolization and

Table 3 Patient analysis according to the type of transcatheter arterial chemoembolization (per patient analysis) *n* (%)

Variable	All treated patients (*n* = 38)	Type of TACE		*P* value
		DEB-TACE (*n* = 22)	c-TACE (*n* = 16)	
Number of nodules per patients	2.2 ± 1.3	2.2 ± 1.2	2.1 ± 1.4	0.7019
Untreated nodules	21/84 (25.0)	13/51 (25.4)	8/33 (24.2)	0.8934
Nodule number class at pathology				0.1473
1 nodule	17 (44.7)	8 (36.4)	9 (56.3)	
1 < nodules < 4	14 (36.8)	11 (50.0)	3 (18.7)	
⩾ 4 nodules	7 (18.4)	3 (13.6)	4 (25.0)	
Sum of tumor diameters (cm)	4.2 ± 2.8	4.1 ± 2.4	4.5 ± 3.3	0.5813
CTA (cm^2)	5.5 (0.8-78.5)	4.6 (1.5-19.1)	7.2 (0.8-78.5)	0.8592
Necrosis on CTA	55.2% ± 37.0%	58.8% ± 36.6%	50.2% ± 38.1%	0.4856
Adherence to MC at pathology				0.4250
Within MC	31 (81.6)	19 (86.4)	12 (75.0)	
Beyond MC	7 (18.4)	3 (13.6)	4 (25.0)	
Histological response				0.2896
Appropriate (necrosis on CTA ⩾ 90%)	11 (28.9)	8 (36.4)	3 (18.7)	
Partial (50% < necrosis on CTA < 90%)	8 (21.1)	3 (13.6)	5 (31.3)	
Inadequate (necrosis on CTA ⩽ 50%)	19 (50.0)	11 (50.0)	8 (50.0)	
Risk factors for recurrence				
Microvascular invasion	7 (18.4)	5 (22.7)	2 (12.5)	0.4271
[1]Grading > 2	7 (18.4)	2/18 (11.1)	5/14 (35.7)	0.1948

Analysis includes the 21 nodules not reached by transarterial treatment (non-targeted lesions). Continuous variables are reported as medians and ranges or means and standard deviations. [1]Assessment of tumor grading was not available for 6 patients who had complete necrosis at explant examination. TACE: Transcatheter arterial chemoembolization; DEB: Doxorubicin-eluting bead; CTA: Cumulative tumor area; MC: Milan Criteria; c-TACE: Conventional TACE.

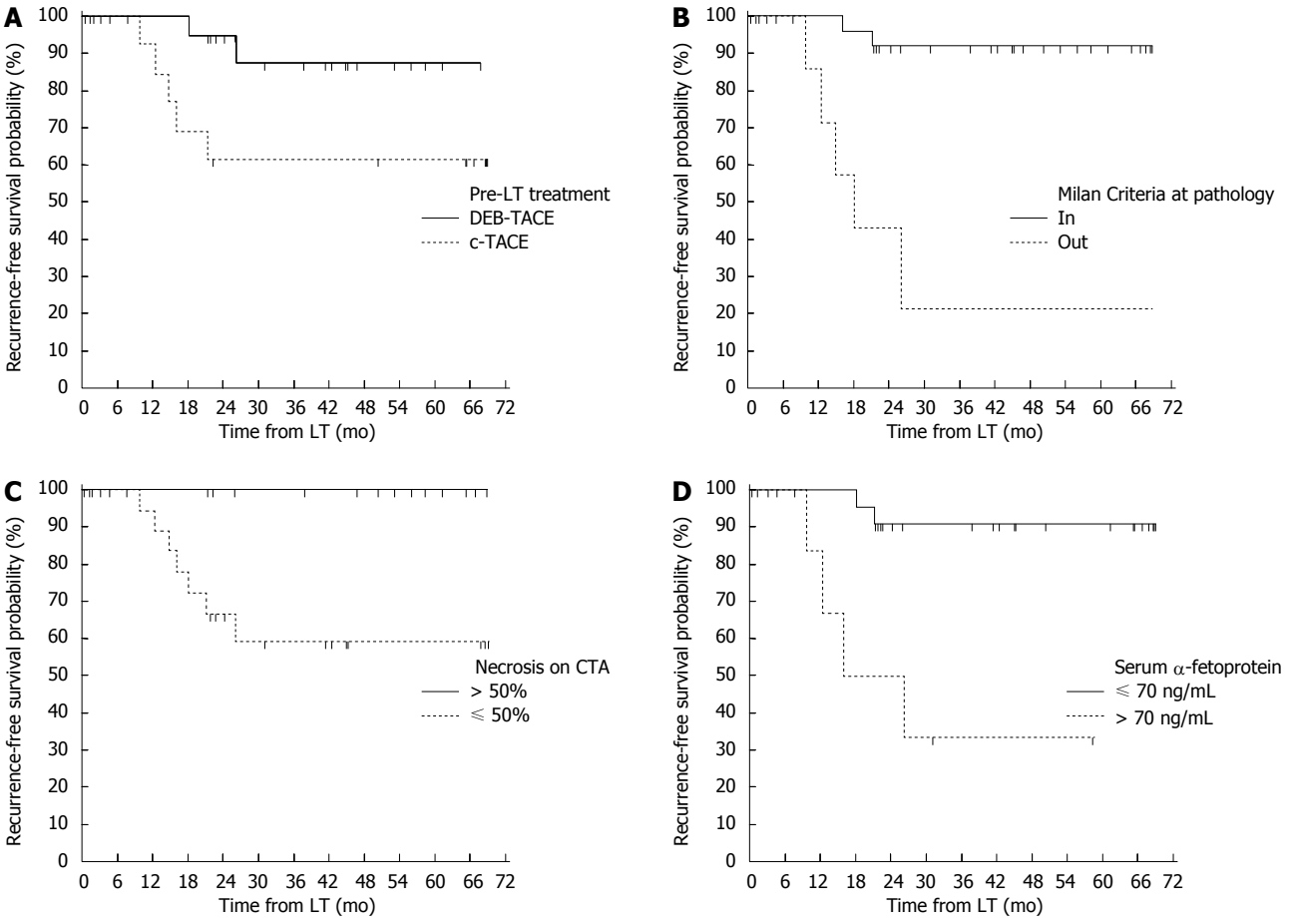

Figure 3 Recurrence-free survival probabilities according to the following. A: Pre-transplant treatment type (log-rank *P* = 0.0493); B: Adherence to Milan Criteria at pathology (log-rank *P* < 0.0001); C: Percentage of necrosis in the cumulative tumor area (log-rank *P* = 0.0098); D: Pre-transplant serum α-fetoprotein level (log-rank *P* = 0.0008). TACE: Transcatheter arterial chemoembolization; DEB: Doxorubicin-eluting bead; LT: Liver transplantation; CTA: Cumulative tumor area; c-TACE: Conventional TACE.

Table 4 Univariate and multivariate analysis of risk factors related to tumor recurrence

Risk factors	Univariate analysis			Multivariate analysis	
	3-yr recurrence-free survival rate	HR (95%CI)	Log rank *P* value	Exp(*b*) (95%CI)	*P* value
Tumor grading G3-G4 (*vs* G1-G2)	66.7% *vs* 74.2%	1.76 (0.289-13.149)	0.4934		
Presence of microvascular invasion (*vs* absence)	60.0% *vs* 80.7%	2.74 (0.451-39.277)	0.2071		
Multiple nodules at pathology (*vs* single)	69.1% *vs* 85.7%	2.15 (0.460-9.070)	0.3478		
Repeated TACE (*vs* single)	74.1% *vs* 80.0%	1.08 (0.244-4.821)	0.9148		
Necrosis on CTA ≤ 50% (*vs* > 50%)	59.3% *vs* 100.0%	NA[1]	0.0098	NA[1]	NA[1]
MC unfulfilled at pathology (*vs* fulfilled)	21.4% *vs* 92.0%	13.84 (10.121-636.416)	< 0.0001	11.6 (1.932-69.646)	0.0077
Absence of DEB-TACE (*vs* presence)	61.5% *vs* 87.4%	4.47 (1.005-22.188)	0.0493	15.45 (1.457-163.766)	0.0237
α-fetoprotein > 70 ng/mL (*vs* ≤ 70 ng/mL)	33.3% *vs* 90.9%	10.31 (4.749-370.242)	0.0008	15.31 (1.766-132.614)	0.0137

[1]Heart rate calculation and the inclusion of the covariate in the multivariate analysis was not applicable due to the lack of hepatocellular carcinoma recurrence events in necrosis on the cumulative tumor area (CTA) > 50% group. TACE: Transcatheter arterial chemoembolization; DEB: Doxorubicin-eluting bead; MC: Milan Criteria; NA: Not available.

increase intra-tumor drug delivery. There are 3 substantial pharmacokinetic advantages associated with DEB-TACE: a continuous elution of the drug for prolonged time, a higher concentration locally into the tumor and a lower systemic exposure to the drug in comparison with c-TACE[12]. In a preclinical study, Hong *et al* demonstrated that the peak of doxorubicin within the tumor is registered after three days, and drug levels remain high up to fourteen days after treatment[14].

Several clinical studies have compared DEB and conventional TACE in non-transplant settings. A recent randomized study including 212 patients failed to demonstrate a significant difference in the overall radiological response, although a better safety profile and a trend toward a better response rate was observed for DEB-TACE[13]. Malagari *et al*[34] demonstrated that DEB-TACE was able to stabilize disease in a higher percentage of patients when compared with bland embolization (embolic agents without drug), but the survival rate at 12 mo did not differ in the two groups. In another prospective study, complete and partial response rates, tumor recurrence and overall survival were similar with DEB-TACE and conventional TACE[35]. Although a retrospective study recently suggested a higher 2-year survival rate in DEB-TACE patients[36], the superiority of this technique remains to be further investigated.

Liver transplant candidates exhibit completely different characteristics than those patients considered in the above-mentioned studies. First, HCC in LT candidates is not advanced. Furthermore, the response to TACE in terms of tumor necrosis has clinical relevance only in those patients who require downstaging, whereas in the others, the goal is to halt tumor progression. Last, recurrence-free survival, measured following LT, is a realistic endpoint, as in the non-transplant setting, TACE is not intended to be curative[37].

Few reports are available about the results of DEB-TACE in LT candidates. A small study from Milan reported a higher complete histological necrosis rate (77%) in patients treated with DEB-TACE. However, only 8 patients had been treated with DEB-TACE in that study, while the 8 patients of the control group received bland embolization (non-loaded microspheres), with very low complete necrosis rates (27.2%)[38]. Unlike from the present study, Farris *et al*[39] recently reported a significantly higher necrosis rate after c-TACE in comparison with DEB-TACE (66.4% *vs* 46.1%). However, no mention of the HCC recurrence-free survival of the patients was made in these studies.

In our series, histopathological examination of the native livers did not indicate a significant difference between DEB-TACE and conventional TACE with regard to the effectiveness of the two different procedures in inducing histological necrosis and achieving tumor downstaging. However, a peculiar histological pattern was associated with DEB-TACE; DEB-TACE was characterized by an intense inflammatory and fibrotic reaction in the area surrounding the tumor tissue that was not observed in those patients treated with conventional TACE. Remarkably, a lower tumor recurrence rate after LT was associated with DEB-TACE. Furthermore, DEB-TACE was identified as an independent predictor of recurrence-free survival in the multivariate analysis. The others independent prognostic determinants found in the present study, serum alpha-fetoprotein levels and adherence to MC at histopathological examination, have been previously identified by others to be strictly linked with HCC recurrence after LT[3].

Pretransplant ablative treatments have the potential to decrease the release and growth of HCC metastases. As the release of tumor cells can be intermittent, the continuous elution of doxorubicin and the distribution of loaded beads in the vessels around the nodule might maintain a prolonged antineoplastic effect and explain the lower recurrence rate observed in the DEB-TACE group. The biological significance of the intense tissue reaction that surrounds the tumor treated with DEB-TACE must to be further investigated. However, one might speculate that the tissue reaction could play a role in limiting tumor spread.

The difference in the chemotherapeutic agent employed in the two different TACE techniques (epirubicin

in c-TACE and doxorubicin in DEB-TACE) is unlikely to have influenced the results; in a large randomized controlled that compared c-TACE made using a lipiodol emulsion containing epirubicin or doxorubicin, no difference in the incidence of adverse reactions, changes in alpha-fetoprotein, extent of tumor reduction or the survival rates between the two drugs was reported[40].

Although further confirmation of our findings with randomized controlled trials is warranted, our report seems to indicate that the use of DEB-TACE in LT recipients with HCC can increase recurrence-free survival after liver transplantation.

COMMENTS

Background

Transcatheter arterial chemoembolization (TACE) is the most common locoregional treatment in cirrhotic patients with hepatocellular carcinoma (HCC) awaiting liver transplantation (LT), and the main objective of TACE is to prevent tumor progression in HCC patients who have already met the Criteria for transplantation or to downstage tumors initially outside Milan Criteria to allow LT. In addition to conventional TACE (c-TACE), based on mixtures of anticancer drugs, lipiodol and a gelatin sponge, a procedure with calibrated doxorubicin-loaded microspheres has been recently developed (DEB-TACE). In pre-LT setting, only a few reports have compared the impact of different TACE regimens on tumor histology and recurrence-free survival after transplantation.

Research frontiers

Pre-clinical and clinical studies have demonstrated that DEB-TACE produces a higher drug concentration within the tumor than does c-TACE in presence of a lower systemic concentration, but its superiority in inducing tumor necrosis and increasing recurrence-free survival remains to be further investigated. As the whole native liver becomes available for histological examination, LT represents a unique setting to correctly assess necrosis and histological changes in tumor nodules of patients treated by TACE. This approach can be useful in developing new strategies to decrease the release of HCC metastases in patients awaiting LT.

Innovations and breakthroughs

A lower tumor recurrence rate after LT was observed in patients who were treated preoperatively with DEB-TACE. Although no significant differences were observed in terms of tumor necrosis between DEB and c-TACE, a peculiar histological pattern was associated with DEB-TACE, characterized by an intense inflammatory and fibrotic reaction in the area surrounding the tumor tissue. This finding, in addition to the prolonged antineoplastic effect of loaded beads in the vessels around the nodule, could limit tumor spread during time on the waiting list and could explain the lower postoperative recurrence rate observed in the DEB-TACE group.

Applications

According to the results of this study, DEB-TACE is an effective locoregional tool for the management of HCC patients awaiting liver transplantation and can increase recurrence-free survival after LT.

Terminology

TACE indicates transcatheter arterial chemoembolization. DEB-TACE indicates TACE with calibrated, doxorubicin-loaded microspheres used to bind, deliver and elute chemotherapeutic drugs in the tumor area. c-TACE indicates the conventional TACE procedure, performed by administering a mixture of epirubicin in an emulsion with lipiodol followed by a gelatin sponge to obtain occlusion of the feeding arteries of the tumor. HCC indicates hepatocellular carcinoma. LT indicates liver transplantation.

Peer review

The authors present an interesting retrospective single-center study that clearly addresses pre-LT treatment of HCC. The authors highlight an important locoregional therapeutic tool, DEB-TACE, which has become increasingly utilized and can improve the outcomes of LT for HCC. The explanted livers underwent very close pathological scrutiny to judge the effects of the 2 different therapies; data analysis is well done. The paper is well written, and the manuscript improves significantly on the knowledge of the role of DEB-TACE in the management of HCC.

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ORIGINAL ARTICLE

Protective effect of naringenin on acetic acid-induced ulcerative colitis in rats

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Abstract

AIM: To evaluate the ameliorative effect of naringenin (NG) during ulcerative colitis (UC) in rats.

METHODS: Rats were treated with three different doses (25, 50 and 100 mg/kg per day) of NG and a single dose of mesalazine (MES, 300 mg/kg per day) for seven days prior to ulcerative colitis induction by 4% acetic acid (AA). Twenty four hours after AA rectal administration, animals were scarified and the colonic tissues were dissected. Colonic mucus content was estimated using Alcian blue dye binding technique. In colon tissues, levels of total glutathione sulphadryls (T-GSH), non-protein sulphadryls (NP-SH) and thiobarbituric acid reactive substances (TBARS) were evaluated. The activities of the antioxidant enzymes, catalase (CAT) and superoxide dismutase (SOD) were measured. Concentrations of nucleic acids (DNA and RNA) and total protein were also estimated in colon tissues. Colonic levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), prostaglandin E_2 (PGE_2) and nitric oxide (NO) were estimated. In cross section of colitis tissue the histopathological changes were observed.

RESULTS: Colonic mucus content was decreased in AA compared to controls (587.09 ± 65.59 mg/kg *vs* 941.78 ± 68.41 mg/kg, $P < 0.001$). AA administration markedly reduced T-GSH (5.25 ± 0.37 nmol/L *vs* 3.04 ± 0.24 nmol/L, $P < 0.01$), NP-SH (3.16 ± 0.04 nmol/L *vs* 2.16 ± 0.30 nmol/L, $P < 0.01$), CAT (6.77 ± 0.40 U/mg *vs* 3.04 ± 0.2 U/mg, $P < 0.01$) and SOD (3.10 ± 0.11 U/mg *vs* 1.77 ± 0.18 U/mg, $P < 0.01$) while TBARS, TNF-α, IL-1β, IL-6, PGE_2 and NO levels (15.09 ± 3.84 nmol/L *vs* 59.90 ± 16.34 nmol/L, $P < 0.01$; 113.56 ± 1.91 pg/mg *vs* 134.24 ± 4.77 pg/mg, $P < 0.01$; 209.20 ± 36.38 pg/mg *vs* 422.19 ± 31.47 pg/mg, $P < 0.01$; 250.83 ± 25.09 pg/mg *vs* 638.58 ± 115.9 pg/mg, $P < 0.01$; 248.19 ± 36.98 pg/mg *vs* 541.74 ± 58.34 pg/mg, $P < 0.01$ and 81.26 ± 2.98 mmol/g *vs* 101.90 ± 10.73 mmol/g, $P < 0.001$) were increased in colon of rats with UC compared controls respectively.Naringenin supplementation, significantly and dose dependently increased the colonic mucus content. The elevated TBARS levels were significantly decreased (39.35 ± 5.86 nmol/L, $P < 0.05$; 26.74 ± 3.17 nmol/L, $P < 0.01$ nmol/L and 17.74 ± 2.69 nmol/L, $P < 0.01$) compared to AA (59.90 ± 16.34 nmol/L) group while the decreased levels of T-GSH and NP-SH and activities of CAT and SOD found increased by NG treatments in dose dependent manner. The decreased values of nucleic acids and total protein in AA group were also significantly ($P < 0.01$) increased in all three NG supplemented groups

respectively. NG pretreatment inhibited the TNF-α levels (123.76 ± 3.76 pg/mg, 122.62 ± 3.41 pg/mg and 121.51 ± 2.61 pg/mg *vs* 134.24 ± 4.78 pg/mg, $P < 0.05$) compared to AA group, respectively. Interleukins, IL-1β and IL-6 levels were also decreased in NG50 + AA (314.37 ± 16.31 pg/mg and 292.58 ± 23.68 pg/mg, $P < 0.05$) and NG100 + AA (416.72 ± 49.62 pg/mg and 407.96 ± 43.87 pg/mg, $P < 0.05$) when compared to AA (352.46 ± 8.58 pg/mg and 638.58 ± 115.98 pg/mg) group. Similar decrease ($P < 0.05$) was seen in PGE_2 and NO values when compared to AA group. The group pretreated with MES, as a reference drug, showed significant ($P < 0.01$) protection against the changes induced in colon tissue by AA administration respectively.

CONCLUSION: In present study, NG produced antioxidant and anti-inflammatory effects demonstrating protective effect in inflammatory bowel disease.

Key words: Naringenin; Ulcerative colitis; Inflammatory bowel disease; Oxidative stress

Core tip: Inflammatory bowel disease (IBD), consisting of Crohn's disease (CD) and ulcerative colitis (UC), results in substantial morbidity and is difficult to treat. New strategies for adjunct therapies are needed. Systemic corticosteroids are highly effective at inducing clinical remission in cases of acute exacerbation of CD and UC; however, their use is limited by their frequent and sometimes severe side effects. Results of present study revealed that, naringenin has protective effects against acetic acid-induced UC by inhibiting inflammatory and oxidative bio-markers. Thus, it may pose promising outcomes for future clinical usage as a natural non-toxic effective supplement in IBD.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a common chronic inflammatory disease of the gastrointestinal tract. Several etiological factors, such as genetic, immunological, and environmental have been linked with the pathophysiology of the disease[1]. There are two main subtypes of IBD; Crohn's disease (CD) and ulcerative colitis (UC) having a combined prevalence of 150-250/100000 population[2,3]. Moreover, the prevalence of hospitalization due to CD and UC is estimated to be 50.1 and 50.6 per 100000 population, respectively[4]. UC involves only the colon and rectum. Although the etiology of UC is not completely understood, it has been commonly associated with reduced antioxidant capacity as well as increased free radical production such as reactive oxygen species (ROS)[5]. Over production of ROS leads to lipid peroxidation (LPO), which can inhibit cellular antioxidant capability finally resulting in prominent colonic inflammation[6]. Clinically, colitis patients were found to overproduce ROS and nitrogen species leading to LPO of membranes and attack on tissue proteins and DNA[7,8]. Endogenous antioxidant defenses against ROS production even in low concentrations influence on two main types: (1) enzymatic such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT); and (2) non-enzymatic such as glutathione (GSH) and ascorbic acid (vitamin C). It is suggested that inflammatory response amplification can induce inflammatory cells chemotaxis resulting in release of ROS and inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-1β, which triggers the pathological responses and symptoms during IBD[9]. Elevated levels of pro-inflammatory cytokines in both the IBD forms reported to have a vital role of such mediators, which also play in determining the severity of the disease[10]. Medications that have ability to inhibit the production of these inflammatory mediators are shown to be clinically effective, which indicate their contribution to IBD and other chronic inflammatory conditions aggravation[11].

Some natural products, such as flavonoids, are getting more attention as novel agents for therapeutically usage. Flavonoids are one of the most abundant natural antioxidants present in plants and the human diets. Naringenin (4,5,7-trihydroxy flavonone) a flavonoid, widely distributed in citrus fruits, tomatoes, cherries, and cocoa[12]. Several pharmacological studies revealed its effects including antidiabetic[13], antiatherogenic[14], antidepressant[15], immunomodulatory[14], antitumor[16], DNA protective[17], hypolipidemic[18] and peroxisome proliferator-activated receptors activator[19]. It has also been shown to have prominent antioxidant[20] and anti-inflammatory[21] potentials. InêsAmaro *et al*[22] reported that, naringenin (NG) has reducing effect on intestinal edema-induced by dextran sodium sulphate in mice.

In several studies, pathogenesis of UC disease has demonstrated that excessive inflammation and oxidative stress play a significant role[23,24]. Amelioration of LPO as well as free radicals scavenging would provide a useful, protective and therapeutic treatment for UC. With respect to the high antioxidant capacity and anti-inflammatory activity, NG would be expected to reduce injury and/or improve tissue healing following injury from ulcerative colitis. In the present study we had evaluated the protective effect of NG during experimental ulcerative colitis and the possible mechanism of action.

MATERIALS AND METHODS

Animals and ethical approval

Eight weeks old male Wister albino rats weighting 250-280 g were received from Experimental Animal

Care Center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. Animals were housed under controlled environmental conditions (25 ℃ and a 12 h light/dark cycle). Animals had free access to Purina rat chow (Manufactured by Grain Silos and Flour Mills Organization, Riyadh, Saudi Arabia) and tap water. All experimental procedures and protocolsin this study including euthanasia were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research (NIH Publications No. 80-23; 1996) as well as the Ethical Guidelines of the Experimental Animal Care Center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia.

Induction of ulcerative colitis

Experimental ulceration in colon tissue was done according to the method described by Mousavizadeh *et al*[25] with slight modification. In brief, under light ether anesthesia rats were administered 2 mL of 4% acetic acid solution (v/v; Merck, Darmstadt, Germany) by transrectally using a (2.7 mm) soft pediatric catheter. After AA administration, rats were holed horizontally for 2 min to prevent AA leakage. Control animals underwent the same procedure using equal volume of normal saline instead of AA solution.

Experimental design

Forty two rats were divided into seven groups (six animals in each) as follows: (1) Control (Cont); (2) NG 100 mg/kg per day (NG100); (3) AA treated rats (AA); (4) NG 25 mg/kg per day + acetic acid (NG25 + AA); (5) NG 50 mg/kg per day + AA (NG50 + AA); (6) NG 100 mg/kg per day + AA (NG100 + AA); and (7) MES 300 mg/kg per day + AA (MES + AA). Naringenin (Sigma Aldrich, United States) and MES treatments were continued for 7 consecutive days by gavage[26]. At end of the treatment, ulcerative colitis was induced in all AA groups. Twenty four hours after the colitis induction, animals were sacrificed under deep anesthesia[27]. The colon (5-6 cm) specimens were dissected, washed with saline solution, imaged, weighted and small cross section was fixed in 10% formaldehyde solution for histopathological evaluation. The remaining tissues were stored at -75 ℃ (Ultra-low freezer, Environmental Equipment, Cincinnati, Ohio, United States) till analysis.

Evaluation of the adherent colonic mucus

The modified procedure of Popov *et al*[28] was used to determine adherent colonic mucus concentration. Briefly, a small portion of colon tissue was excised, weighted then transferred immediately to 1% Alcian blue solution (in 0.16 mol/L sucrose solution buffered with sodium acetate, pH 5) for 24 h. The excess dye was removed by rinsing with sucrose solution. The dye complexed with the gastric wall mucus was extracted using 10 mL of 0.5 mol/L $MgCl_2$ solution. A 4 mL aliquot of blue extract was then shaken with an equal volume of diethyl ether. The resulting emulsion was centrifuged at 4000 rpm and the absorbance of the aqueous layer was recorded at 580 nm by using spectrophotometer (LKB-Pharmacia, Mark II, Ireland). The quantity of Alcian blue extracted (μg) per grams of wet colonic tissue was then calculated.

Histopathological investigations

Colon sections were fixed 10% neutral buffered formalin then put for 24 h in decal. Samples were then cut into several sections and embedded into paraffin wax blocks. Tissues were stained with haematoxylin and eosin and were mounted and observed microscopically for histopathological changes by a pathologist in blinded fashion.

Estimations of total glutathione sulphadryls and non-protein sulphadryls concentrations in colon

In colon tissues, total glutathione sulphadryls (T-GSH) and non-protein sulphadryls (NP-SH) levels were estimated according to the method described by Sedlak *et al*[29]. Tissues were homogenated in ice-cold 0.02 mol/L ethylenediamine tetra-acetic acid. An aliquots of 0.5 mL of tissue homogenate was mixed with 0.2 mol/L Tris buffer, pH 8.2 and 0.1 mL of 0.01 mol/L Ellman's reagent, [5,5'-dithiobis-(2-nitr-benzoic acid)] (DTNB). Each sample tube was centrifuged at 3000 rpm at room temperature for 15 min the absorbance of the clear supernatant was measured using spectrophotometer (LKB-Pharmacia, Mark II, Ireland) at 412 nm. For NP-SH estimation, homogenate was diluted with distilled H_2O and mixed with 1 mL of 50% trichloroacetic acid (TCA). The tubes were shaken intermittently for 10-15 min and centrifuged for 15 min at approximately 3000 *g*. Two milliliter of supernatant was then added to 4 mL of 0.4 mol/L Tris buffer (pH 8.9) then 0.1 mL DTNB added. The absorbance was read within 5 min of the addition of DTNB at 412 nm against a reagent blank.

Estimation of thiobarbituric acid reactive substances levels in colon

A thiobarbituric acid reactive substances (TBARS) assay kit (ZeptoMetrix, United States) was used to measure the LPO products, malondialdehyde (MDA) equivalents. Briefly, one hundred microliters of colon homogenate was added to 2.5 mL reaction buffer (provided by the kit) and heated at 95 ℃ for 60 min. After the mixture cooling, supernatant absorbance was measured at 532 nm using a spectrophotometer (LKB-Pharmacia, Mark II, Ireland). The LPO products are expressed in terms of nmoles MDA/mg protein.

Estimation of CAT and SOD activities in colon

Catalase activity in colon tissues was estimated by the method described by Aebi[30]. In brief, aliquot of 0.5 mL postmitochondrial supernatant was mixed with 2.5 mL of 50 mmol/L phosphate buffer (pH 7.0) and 20 mmol/L H_2O_2. CAT activity was estimated spectrophotometrically following the decrease in absorbance at 240 nm and expressed in terms of units/mg protein as compared to a standard curve.

The SOD activity in colon tissue was measured by using the method described by Kono[31]. The principle of this method was that superoxide anions generated by the

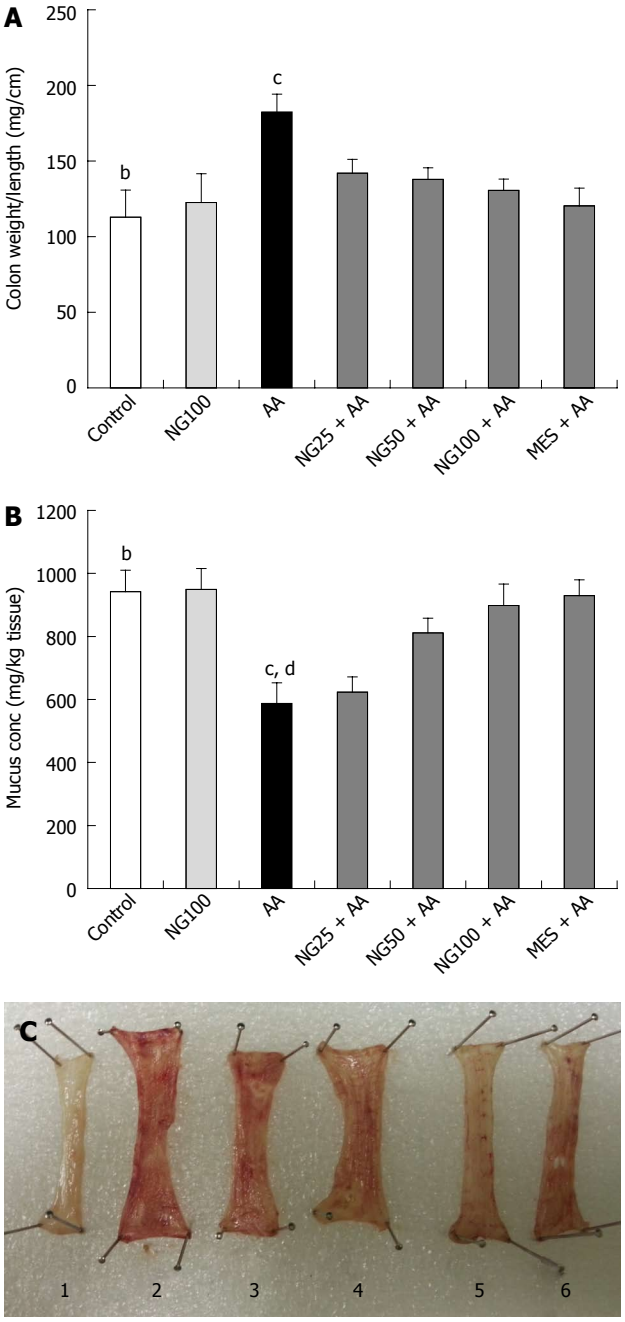

Figure 1 Effect of naringenin on colon weight/length (A), mucus concentration (B) and induction of ulceration and its protection by treatments in colonic tissue of rats with acetic acid-induced ulcerative colitis (C) (*n* = 6). Values in (A) and (B) are expressed as mean ± SE and analyzed using one way analysis of variance followed by Newman-Keuls *post hoc* test. $^{b}P < 0.01$ control *vs* acetic acid (AA); $^{c}P < 0.05$, $^{d}P < 0.01$ AA *vs* naringenin (NG) 25 + AA, NG50 + AA, NG100 + AA or mesalazine (MES) + AA groups. Groups in (C) are arranged as follows: control (1), AA (2), NG25 + AA (3), NG50 + AA (4), NG100 + AA (5) and MES + AA (6).

oxidation of hydroxylamine hydrochloride can mediate the reduction of nitrobluetetrazolium to blue formazon. The color was then measured at 560 nm under aerobic conditions. Addition of superoxide dismutase inhibited nitrobluetetrazolium reduction and the extent of this inhibition was taken as a measure of enzymatic activity. The SOD activity was expressed as units/mg protein.

Determination of nucleic acids and total protein levels in colon

The method described by Bregman[32] was used to determine the levels of nucleic acids (DNA and RNA) in colon. In brief, colon tissues were homogenized in 4 mL ice-cold distilled water and 2 mL homogenate was suspended in 5 mL of 10% ice-cold trichloroacetic acid (TCA). After centrifugation, the pellet was extracted twice with 95% ethanol. Finally, the nucleic acids were extracted in 5% TCA. DNA was determined by treating the nucleic acid extract with diphenylamine reagent and measuring the intensity of blue color at 600 nm. For quantification of RNA, the nucleic acid extract was treated with orcinol reagent and the green color was read at 660 nm. Standard curves were used to determine the amounts of nucleic acids present. Total protein in colon was estimated by Lowry *et al*[33] method using Bovine plasma albumin as a standard.

Determination of inflammatory cytokines, PGE_2 and NO levels in colon

In colon, TNF-α, IL-1β, IL-6 and PGE_2 levels were assessed and quantified according to the method of-Mousavizadeh *et al*[25] using enzyme-linked immunoabsorbent assay ELISA (R and D systems, United States). The results were expressed as pg/mg tissue. Levels of colonic nitric oxide were assayed by Griess reaction method using commercial kit (R and D systems, United States).

Statistical analysis

Data were expressed as mean ± SE. Statistical analysis was carried out using one-way ANOVA followed by Newman-Keuls *post hoc* test. *P* value of $\leqslant 0.05$ was considered statistically significant. All statistics tests were conducted using Graph Pad Prism (version 5) software.

RESULTS

Acetic acid significantly ($P < 0.01$) increased the colonic weight as compared to control group. Pretreatment with NG following three doses and MES for 7 d, showed significant ($P < 0.05$) inhibition in weight increase while compared to AA group (Figure 1A). Mucus concentration was significantly ($P < 0.01$) reduced in AA administered group when compared to control animals. Pretreatment with higher doses (50 and 100 mg/kg) of NG and MES significantly elevated the reduced colonic mucus concentration ($P < 0.05$, $P < 0.01$ and $P < 0.05$, respectively) as compared to AA group (Figure 1B). The colon images were clearly showed the induction of ulceration and its protection by the treatments (Figure 1C).

Histopathological changes with their intensity are presented in Figure 2. Slide from control group, showing benign mucosal epithelium of tall columnar epithelial cells with goblet cells (Table 1 and Figure 2A). In the AA group, the slide revealed diffused active colitis with widely eroded mucosa with ulcerations and necrosis associated with edema, goblet cell hyperplasia, lymphoid follicular

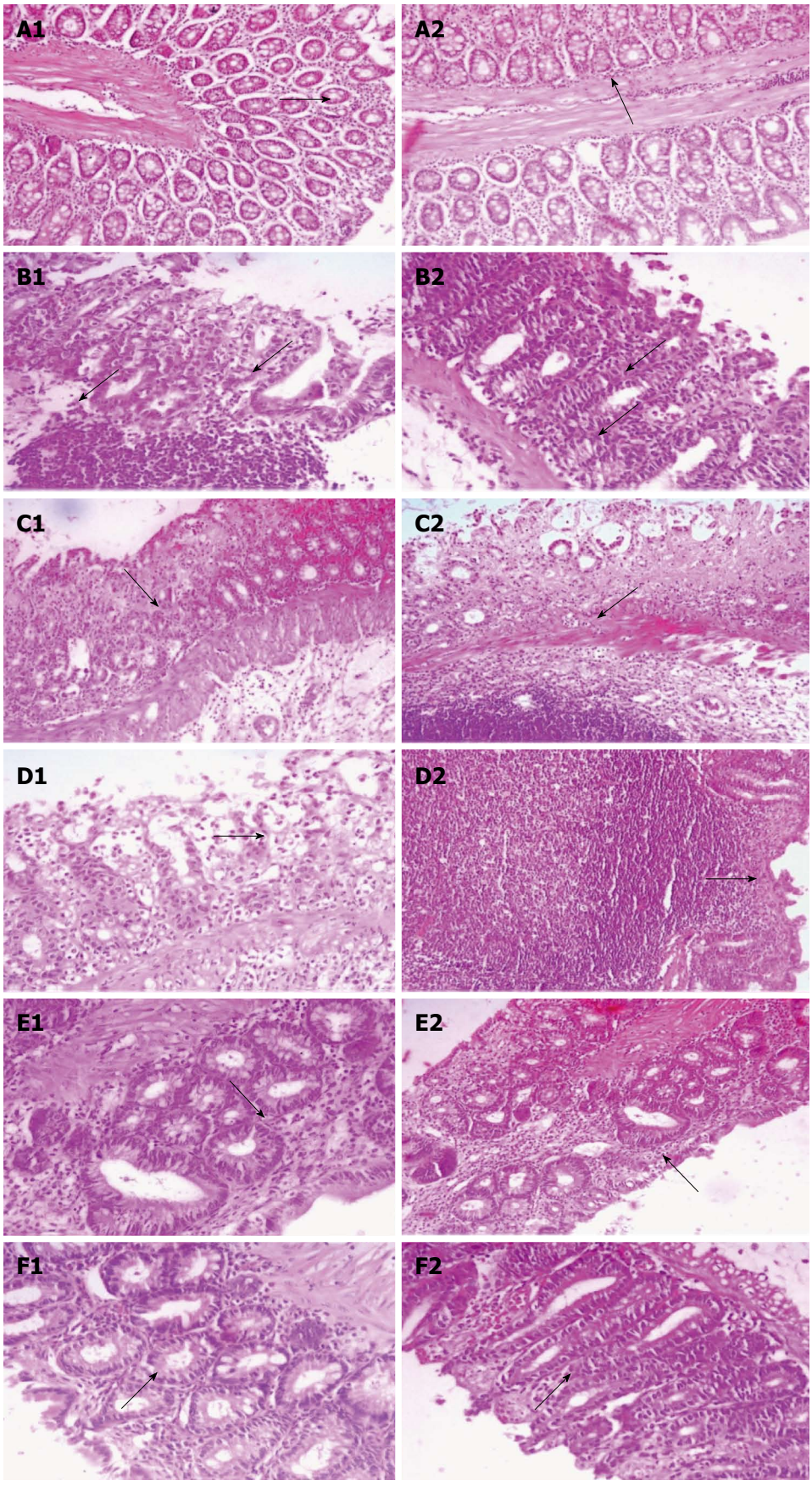

Figure 2 Histopathological changes with their intensity are presented. A-1 and 2: Histopathological colonic sections showing normal benign looking mucosa; B-1 and 2: Diffused active colitis with superficial erosions, stormal edema, dense acute and chronic inflammatory cells infiltrate with widely ulcerating mucosa; C-1 and 2: Reparative epithelial changes with little ulcer healing and inflammatory cells infiltrate; D-1 and 2: Reparative epithelial changes and healing ulcer with lymphoid follicle form; E-1 and 2: Healing ulcer and reparative epithelial changes; F-1 and 2: Attenuated cell damage with complete ulcer healing. A1-F1 (× 400), A2-F2 (× 200).

Table 1 Effect of naringenin on microscopic scoring of histopathological sections of colonic tissue of rats with acetic acid-induced ulcerative colitis

Groups	Ulceration	Hyperemia	Necrosis	Edema	Cellular infiltrate	Goblet cell hyperplasia
Control	0	0	0	0	0	0
AA	+++	+++	++++	+++	++++	++
NG25 + AA	++	++	+++	++	+++	++
NG50 + AA	++	++	++	++	+++	+
NG100 + AA	+	+	+	+	++	+
MES + AA	+	+	+	+	+	+

MES: Mesalazine; AA: Acetic acid; NG: Naringenin; NG25: NG 25 mg/kg per day; NG50: 50 mg/kg per day; NG100: 100 mg/kg per day; 0: Normal; +: Mild; ++: Moderate; +++: Sever; ++++: Very sever.

hyperplasia and transmural lymphoplasmacytic infiltrate with few intraepithelial neutrophilic cells within stromal (Table 1 and Figure 2B). In NG25 + AA group, slight healing epithelial cells with scattered superficial ulcers lined by colonic glands with reparative epithelial changes with hyperchromatic nuclei and infrequent mitosis and less goblet cells surrounded by transmucosal fewer lymphoplasmacytic infiltrate within stromal edemawas seen (Table 1 and Figure 2C). Slide from NG50 + AA group showed intestinal rat lined by healing epithelial cells with tall columnar epithelium, with superficial shredded epithelial cells, less eroded surface surrounded by few inflammatory edema and less necrosis with colonic gland showed reparative epithelial changes (Table 1 and Figure 2D). In higher dose of NG treatment (NG100 + AA) group, superficial tiny eroded mucosa with mucosal, hemorrhage, edema and scattered acute and chronic inflammatory cells infiltrate surrounding colonic glands with reparative epithelial changes and few goblet cells were seen (Table 1 and Figure 2E). Slide from MES + AA group revealed intestinal section with more better healed and improvement of intestinal mucosa compared to positive controlled sections with few mucosal lymphoplasmacytic infiltrate within stromal edema (Table 1 and Figure 2F).

Acetic acid administration resulted in a significant ($P < 0.01$) decrease in colon levels of both T-GSH and NP-SH when compared to control animals. Pretreatment with NG with higher doses (50 mg/kg and 100 mg/kg) significantly ($P < 0.01$) attenuated T-GSH and NP-SH ($P < 0.05$) the reduced levels as compared to AA group. Pretreatment with MES significantly inhibited the decreased levels of T-GSH and NP-SH ($P < 0.001$ and $P < 0.05$, respectively) (Figure 3A and B). Concentration of TBARS in the colons of AA treated rats were significantly ($P < 0.01$) increased compared to control animals. A significantly lower concentrations of TBARS values were found in NG25 + AA ($P < 0.05$), NG50 + AA ($P < 0.01$), NG100 + AA ($P < 0.01$) and MES + AA ($P < 0.01$) groups as compared to AA group (Figure 3C). CAT activity was significantly ($P < 0.01$) decreased in colon tissues of AA administered rats compared to control animals. Pretreatment with 50 mg/kg per day and 100 mg/kg per day of NG, significantly ($P < 0.05$) inhibited the decrease CAT activity in colon as compared to AA group (Figure 3D). SOD activity was significantly ($P < 0.01$) reduced in the colons of AA treated animals as compared to control rats. Group of rats pretreated with 100 mg/kg per day of NG for 7 d showed a significant ($P < 0.05$) increase in colon SOD activity while compared to AA treated animals (Figure 3E). Pretreatment with MES also markedly ($P < 0.05$ and $P < 0.01$, respectively) enhanced the CAT and SOD activities as compared to AA group (Figure 3D and E).

There was a significant ($P < 0.001$) decrease in colon levels of DNA, RNA and total protein in AA administered group as compared to control animals. Pretreatment with NG (100 mg/kg per day) or MES (300 mg/kg per day) significantly ($P < 0.01$) increased the DNA content in colon tissue compared to AA group. The RNA levels in NG higher doses and MES groups found significant ($P < 0.01$) elevation compared to AA group. Total protein levels were also significantly ($P < 0.05$) increased in NG50 + AA, NG100 + AA and MES + AA groups compared to AA group (Table 2).

Pro-inflammatory cytokines including TNF-α, IL-1β and IL-6 levels produced significant ($P < 0.01$) increase in AA-induced ulcerative colitis and levels found significantly ($P < 0.05$ and $P < 0.01$) diminished in NG higher doses and MES pretreated groups as compared to AA group, respectively (Figure 4A-C). Similar changes in PGE_2 levels were seen in colon tissue of rats (Figure 4D). In colon tissue, NO levels were significantly ($P < 0.01$) increased AA group compared to controls. The elevated NO levels were markedly ($P < 0.05$) reduced in NG and MES treated group compared AA group respectively (Figure 4E).

DISCUSSION

Present investigation outlines the anti-ulcerogenic effect of NG against experimentally induced UC in rats as a model for IBD. The preventative effect of NG was confirmed by histological evaluation and also using MES as a standard drug. Seven days pretreatment with NG significantly reduced the AA-induced colonic mucus content and prevented oxidative and inflammatory response in dose dependent manner.

Ulcerative colitis is characterized by mucosal inflammation and ulcerations with a variable extent and severity[34]. Rectal administration of 4% AA to experimental rodents to induce UC is a well-established animal model, which phenotypically resembles human colon inflammation[35]. It also causes colonic epithelial lesions and

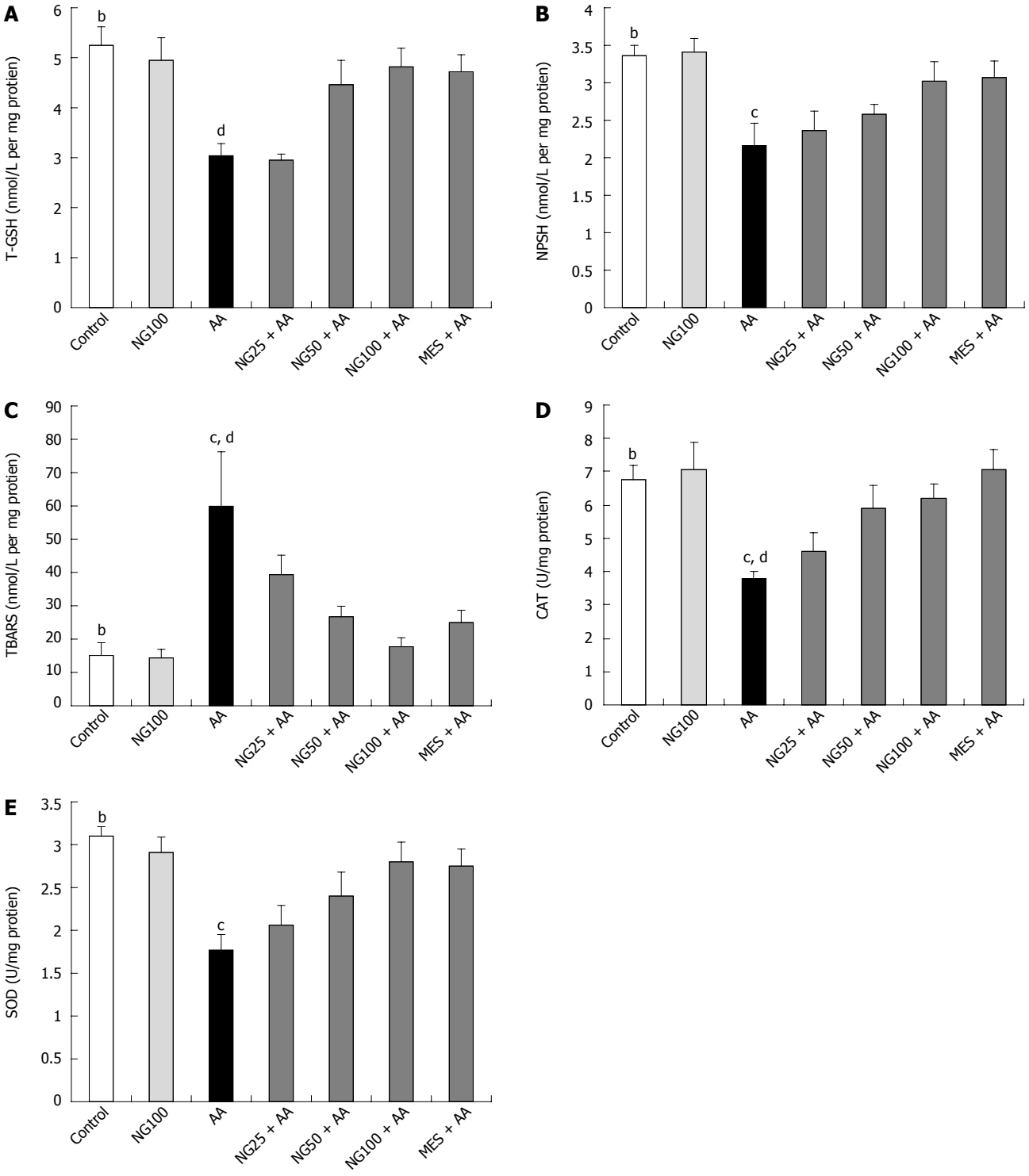

Figure 3 Effect of naringenin on total glutathione sulphadryls (A), non-protein sulphadryls (B) and thiobarbituric acid reactive substances levels (C) as well as catalase (D) and superoxide dismutase activities (E) in colonic tissue of rats with acetic acid-induced ulcerative colitis (*n* = 6). Values are expressed as mean ± SE and analyzed using one way analysis of variance followed by Newman-Keuls *post hoc* test. $^{b}P < 0.01$ control *vs* AA; $^{c}P < 0.05$, $^{d}P < 0.01$ AA *vs* NG25 + AA, NG50 + AA, NG100 + AA or MES + AA groups. T-GSH: Total glutathione sulphadryls; NP-SH: Non-protein sulphadryls; TBARS: Thiobarbituric acid reactive substances; CAT: Catalase; SOD: Superoxide dismutase; AA: Acetic acid; UC: Ulcerative colitis; MES: Mesalazine; NG: Naringenin.

necrosis associated with neutrophils and macrophages infiltration to the damaged colon indicating inflammatory conditions[28,35]. In present study, the 4% AA administration resulted a significant increase in colonic weight and induced sever ulceration and tissue necrosis associated with inflammatory infiltrate and goblet cell hyperplasia as indicated in the results of the histopathologicalestimations. Similar pathological impairments were reported in earlier studies using the same animal model[26,36]. Application of AA in the present study disturbed the colonic mucus, which is in agreement with Popov *et al*[28]. Colonic mucus plays an important protective role against

Table 2 Effect of naringenin on DNA, RNA and total protein levels in colonic tissue of rats with acetic acid-induced ulcerative colitis

Groups	DNA (μg/100 mg wet tissue)	RNA (μg/100 mg wet tissue)	Total protein (mg/100 mg wet tissue)
Control	652.05 ± 17.12[b]	378.51 ± 38.44[b]	3.0 ± 0.19[b]
NG100	688.84 ± 47.69	380.24 ± 48.96	2.43 ± 0.31
AA	222.69 ± 18.78[d]	167.35 ± 15.16[d]	0.95 ± 0.08[c]
NG25 + AA	293.93 ± 33.49	208.68 ± 25.05	1.32 ± 0.13
NG50 + AA	338.54 ± 21.44	250.06 ± 10.11	2.02 ± 0.46
NG100 + AA	415.50 ± 41.51	298.34 ± 12.92	2.15 ± 0.27
MES + AA	425.04 ± 38.23	307.44 ± 18.31	2.14 ± 0.26

Values are expressed as mean ± SE (n = 6) and analyzed using one way ANOVA followed by Newman-Keuls *post hoc* test. [b]P < 0.01 control *vs* acetic acid (AA); [c]P < 0.05, [d]P < 0.01 AA *vs* Naringenin (NG) 25 + AA, NG50 + AA, NG100 + AA or mesalazine (MES) + AA groups.

chemically induced ulceration which may also facilitate the repair of the damaged epithelium[37]. Although, numerous pharmacotherapies have been suggested for UC treatment, the side effects or toxicity of these medications are a major clinical problem[38]. That is why naturally occurring products such as flavonoids are now suggested as an alternative option beside the conventional therapies[39]. Indeed, earlier experimental studies demonstrated flavonoids such as quercitrin, kushenin, kaempferol and baicalin to promote UC healing[40-43].

Previous studies demonstrated that NG administration effectively protected the experimentally induced gastric lesions and ulcers[44,45]. Protection against experimental UC induced by NG was accompanied by restoration of the increased colon thickening in AA group, which is an indirect assessment of colon inflammation. Microscopic scoring of the histopathological sections confirmed the protective action of NG as it decreased colonic tissue ulceration, necrosis and inflammation in dose dependent manner. Motilva *et al*[45] reported that NG treatment increased the gastric mucus levels in rats induced gastric lesions by absolute ethanol. In the present study, NG was also found to inhibit the depletion of colonic mucus caused by AA treatment. This protective activity could be attributed to its antioxidant and anti-inflammatory properties.

Oxidative stress is known to play an important role in IBD initiation and progression[46]. Experimentally induced colitis in animals is characterized by oxidative damage and an imbalance between oxidant and antioxidant substances[47]. The AA-induced colitis model is known to cause vascular dilatation and white blood cells accumulation, as well as an increase in blood flow, leading to increased production of oxygen and hence the excessive generation of free radical and ROS[35,48]. Several studies have indicated the vital role that free radicals play in the pathogenesis of mucosal injuries[49,50]. Moreover, free radicals and ROS were reported in colorectal specimens of ulcerative colitis[51,52]. The first line of oxidative defense system against free radicals is the sulphadryls groups in peptide namely GSH or NP-SH. It is widely distributed in all biological tissues and work as a non-enzymatic antioxidant. GSH inhibits ROS oxidative injuries directly *via* its sulfhydryl group and indirectly as a cofactor or a coenzyme in ROS enzymatic detoxification process[53,54]. Another line in oxidative defense system is the enzymatic antioxidants. Examples for important antioxidant enzymes are SOD, CAT, and GPx[55]. In present study, levels and activities of non-enzymatic and enzymatic defense systems were severely decreased in the colon of AA administered animals indicating oxidative cellular injury. Furthermore, free radicals are known to attack lipid contents of cellular membranes leading to activation of LPO process and cellular damage. Therefore, the concentrations of LPO specific products such as TBARS were elevated, while the critical cellular macro- and micro-molecules such as nucleic acids and total proteins levels were decreased in the present work indicating cellular oxidative injury and cytotoxicity. These results, which are in agreement with previous findings, suggest the harmful effects of AA on cellular macromolecules and its ability to impair the epithelial cell integrity and hinder mucosal recovery[23,36].

In present study, NG was able to attenuate AA induced oxidative damage and injury of colon tissues confirming its strong antioxidant and anti-inflammatory properties. It has seen in earlier studies that NG markedly increased the antioxidant markers such as GSH, NP-SH levels and SOD, and CAT activities[12,56,57]. Han *et al*[56] found that NG pretreatment can increase the activity of antioxidant enzyme GPx, which suggest the ability of NG to attenuate oxidative stress by decreasing the lipid peroxide level and to inhibit accumulation of free radicals generation during LPO process[57]. In the current study, NG treatment significantly corrected the impaired levels of nucleic acids and total protein in colon tissue suggesting the cytoprotective properties of the naturally occurring flavonoids. The antioxidant activity of NG depends mainly on the presence of B-ring catechol group, which can stabilize a radical species by donating hydrogen (H^+)[12].

Inflammatory cytokines are known to play a crucial role in modulating mucosal immune system where the neutrophils and macrophages are responsible for disrupting epithelial integrity and causing colon injury[58]. The pathogenesis of UC is characterized by migration of granulocytes and other leukocytes to the inflamed mucosa and superficial ulcers leading to increased levels of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β[59,60]. In present study, the elevated colon level of TNF-α, IL-6 and IL-1β in AA administered group is an evidence for epithelial cell necrosis, edema, and neutrophil infiltration, which is also supported by the histopathological results. These results are in accordance with earlier experimental and clinical studies[6,28,36,61]. The reported increased levels of colonic PGE_2 in AA group of animals is in agreement with Otani *et al*[62], where the enhanced level of PGE_2 was attributed to its overproduction rather than decreased metabolism, both of which are mediated by pro-inflammatory cytokines. Naringenin was found in the current

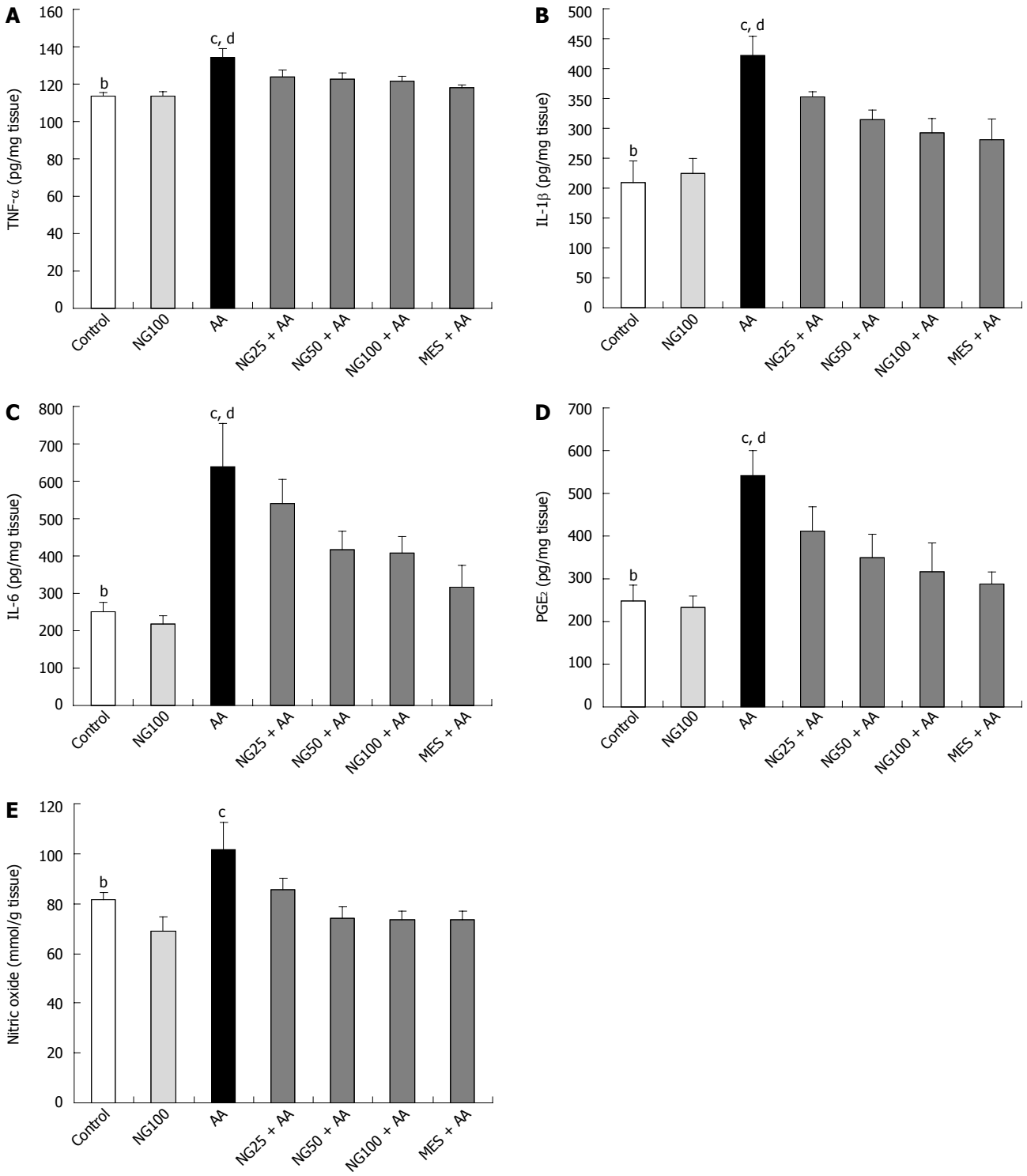

Figure 4 Effect of naringenin on tumor necrosis factor-α (A), interleukin-1β (B), interleukin-6 (C), prostaglandin E_2 (D) and nitric oxide (E) levels in colonic tissue of rats with acetic acid-induced ulcerative colitis (*n* = 6). Values are expressed as mean ± SE and analyzed using one way analysis of variance followed by Newman-Keuls *post hoc* test. $^{b}P < 0.01$ control *vs* AA; $^{c}P < 0.05$, $^{d}P < 0.01$ AA *vs* NG25 + AA, NG50 + AA, NG100 + AA or MES + AA groups. TNF-α: Tumor necrosis factor-α; IL-1β: Interleukin-1β; IL-6: Interleukin-6; PGE_2: Prostaglandin E_2; AA: Acetic acid; NG: Naringenin; MES: Mesalazine.

and earlier studies to inhibit the level of inflammatory cytokines including TNF-α, IL-6 and IL-1β[63]. The anti-inflammatory properties of naringenin were suggested to be through several mechanisms including increased phosphorylation of ERK 5 and P38 MAPK and inhibition of NF-κB and activator protein-1 signaling[64,65]. Additionally, naringenin, which present in high concentrations in citrus fruits, was found to block NF-κB activation resulting in down regulation of the downstream target genes of NF-κB such as iNOS and COX-2 expression[66]. These enzymes catalyze oxidative stress-induced production of NO and prostaglandins respectively, which are known as an important inflammatory mediators in the pathogenesis of colitis[63,67]. These findings are in agreement with our

results where pretreatment with naringenin significantly ameliorated AA induced elevation of the level of PGE_2 and NO in rats' colon.

In conclusion, the present study revealed that NG-protects the AA-induced ulcerative colitis by inhibiting inflammatory and oxidative bio-markers. Finally, our results may pose promising outcomes for future clinical usage of NG as a natural non-toxic effective supplement in IBD.

COMMENTS

Background

The pathogenesis of inflammatory bowel disease (IBD) such as ulcerative colitis (UC) is usually associated with reduced antioxidant capacity. Generation of free radicals like reactive oxygen species (ROS) leads to lipid peroxidation, which inhibits cellular antioxidant capability, resulting in prominent colonic inflammation. There is a great need to search for safe and tolerable compounds for the management of IBD to reduce patient compliance as well as the adverse effects of conventional treatments. Naringenin (NG) is a naturally occurring flavonoid that can be extracted from citrus fruits, tomatoes, cherries, grapefruit, and cocoa. Like most of the flavonoids, NG was experimentally found to have several pharmacological potentials, including antioxidant, antitumor and anti-inflammatory because of NG has properties to produce sufficient hydroxyl (-OH) substitutions, which give it the capability to scavenge ROS. Thus, it has considered that NG may diminish and/or improve pathological conditions where oxidation or inflammation is deemed to play a vital role, like in case of IBD.

Research frontiers

In the present study, NG was orally (gavage) treated with three doses (25, 50 and 100 mg/kg per day body weight) for 7 consecutive days, 24 h later UC was induced by 4% acetic acid. In colitis tissue, Alcian blue, pro-oxidative and inflammatory biomarkers were estimated. The biochemical alterations were further justified with histopathological changes.

Innovations and breakthroughs

NG pretreatment clearly revealed the protection against acetic acid-induced UC in animal model. Antioxidant and anti-inflammatory properties of NG are suggested to be the key for these effects as NG significantly reduced oxidative stress and inflammatory biomarkers in a dose dependent manner.

Applications

The present data shows that NG has a promising protective effect against experimentally-induced UC in animal model. Thus, NG could be recommended for its use as potential alternative and complementary therapy for IBD after confirmation of the obtained findings by clinical trials.

Peer review

The preclinical preventative properties of NG against UC are outlined in present study. Also the possible pharmacological mechanisms of action responsible for these effects are evaluated. Overall, this study proofed that NG is an effective and safe compound that worth to be investigated in future clinical trials for its colonic anti-ulcerogenic properties.

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BRIEF ARTICLE

Quality of compounded topical 2% diltiazem hydrochloride formulations for anal fissure

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Abstract

AIM: To investigate the quality of topical 2% diltiazem formulations extemporaneously compounded by retail pharmacies openly offering drug-compounding services.

METHODS: A participating healthcare professional wrote 12 prescriptions for compounded 2% diltiazem cream, with 2 refills allowed per prescription. The 12 sets of prescriptions were filled, at intervals of 1-2 wk between refills, at 12 different independent retail pharmacies that openly offer drug-compounding services in a major metropolitan region. The 36 resultant preparations, provided as jars or tubes, were shipped, as soon as each was filled, at ambient temperature to the study core laboratory for high-performance liquid chromatography (HPLC) analysis, within 10 d of receipt. For the HPLC analysis, 8 different samples of the topical diltiazem, each approximately 1 g in weight, were taken from prespecified locations within each container. To initiate the HPLC analysis, each sample was transferred to a 100 mL volumetric flask, to which methanol was added. The HPLC analysis was conducted in accordance with the laboratory-validated method for diltiazem in cream, ointment, and gel formulations. The main outcome measures were potency (percentage of label claim) and content uniformity of the compounded topical 2% diltiazem formulations.

RESULTS: Of the 36 prescriptions filled, 30 were packaged in jars and 6 were packaged as tubes. The prescriptions were specifically for cream formulations, but 6 of the 12 pharmacies compounded 2% diltiazem as an ointment; for another pharmacy, which had inadequate labeling, the dosage form was unknown. The United States Pharmacopoeia (USP) standard for potency is 90%-115% of label claim. Of the 36 preparations, 5 (13.89%) were suprapotent and 13 (36.11%) were subpotent. The suprapotent prescriptions ranged in potency from 117.2% to 128.5% of label claim, and the subpotent prescriptions ranged in potency from 34.8% to 89.8% of label claim. Fourteen (38.9%) preparations lacked content uniformity according to the USP standard of 90%-110% potency and < 6% relative standard deviation. Of the 30 formulations packaged in jars, 12 (40%) lacked content uniformity, while of the 6 formulations packaged in tubes, 2 (33.3%) lacked content uniformity. Nine of the 12 pharmacies (75%) failed USP potency or content-uniformity specifications for at least 1 of the 3 prescription fills. For 5 of the 12 pharmacies (41.7%), the mean potency across all three prescription fills was < 90% of label claim.

CONCLUSION: Patients prescribed topical 2% diltiazem for treatment of anal fissure frequently receive compounded formulations that are misbranded with respect to potency and that lack content uniformity.

Key words: Anal fissure; Pharmacy compounding; Topical diltiazem; Formulation potency; Content uniformity

Core tip: The use of topical 2% diltiazem hydrochloride for treating anal fissures is supported by multiple clinical trials and is recommended in published practice parameters. As no commercially manufactured formulation of topical 2% diltiazem has been approved yet by the Food and Drug Administration for the treatment of anal fissure, prescriptions for the medication need to be extemporaneously compounded by retail pharmacies. Employing high-performance liquid chromatography analysis of topical 2% diltiazem formulations compounded by a sampling of pharmacies, we found a notable trend toward lack of content uniformity and misbranding of potency, suggesting that many patients might not receive the anticipated relief of anal-fissure pain.

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INTRODUCTION

The use of topical 2% diltiazem hydrochloride for treating anal fissures by lowering anal sphincter pressure has been explored in multiple clinical trials since 2000[1-5]. Diltiazem hydrochloride, a calcium channel blocker, is well known as an oral treatment for hypertension and angina[6]. In 2010, the Standard Practice Task Force of the American Society of Colon and Rectal Surgeons (ASCRS) published revised practice parameters for managing anal fissure, assigning to each practice parameter a grade of recommendation and a class of evidence[7]. Noting that conservative (nonsurgical) therapy is safe and should be the first step for managing anal fissure[8-10], the ASCRS task force stated that topical formulations of calcium channel blockers may be appropriately used to treat anal fissure and that these drugs seemed to have a lower incidence of adverse effects than topical nitrates, such as nitroglycerin. This practice parameter was accorded the highest grade of recommendation and the second highest class of evidence by the task force[7].

No commercially manufactured formulation of topical 2% diltiazem has been approved by the United States Food and Drug Administration (FDA) for the treatment of anal fissure. Consequently, colon and rectal surgeons, gastroenterologists, and other physicians who want to follow ASCRS practice parameters and prescribe a topical calcium channel blocker for treatment of anal fissure have to write prescriptions for a product that will be extemporaneously compounded by retail pharmacies. Directions for compounding 2% diltiazem as a topical formulation are readily available in published literature. For example, propylene glycol, hydroxyethyl cellulose, and heated purified water are mixed with diltiazem, and the resulting formulation is packaged in a tight, light-resistant container, usually a tube or jar[11]. A few pharmacies that specialize in compounding services advertise the availability of compounded topical 2% diltiazem on the internet. However, many nonspecialized retail pharmacies also fulfill prescriptions by compounding the product.

In 2006, the FDA investigated the quality of compounded products, collecting active pharmaceutical ingredients (API) and finished compounded drug samples during unannounced visits to compounding pharmacies throughout the country. All API samples passed analysis, but a third of the 36 compounded samples that were collected failed analysis by being either subpotent or supra-potent or by lacking content uniformity. The United States Pharmacopoeia (USP) standard for potency is 90%-115% of label claim[12]. Because the API samples passed analysis, the FDA observed that the failures of the samples in the analysis were directly related to faulty compounding processes at the pharmacies, including the lack of proper in-process controls and end-product testing[13].

To examine the quality of compounded formulations of topical 2% diltiazem, we undertook a high-performance liquid chromatography (HPLC) analysis of preparations gathered from retail pharmacies in a major metropolitan region.

MATERIALS AND METHODS

Data source

A healthcare professional was asked to write prescriptions for extemporaneously compounded 2% diltiazem cream for fulfillment by retail pharmacies in the greater New York metropolitan region. The selection criteria, intended to locate retail pharmacies that might have experienced pharmacists on staff with competency at compounding, included stipulations that the pharmacies be independent, not parts of retail pharmacy chains, and that they openly offer drug-compounding services by means of online or other advertising. A total of 12 qualifying retail pharmacies were selected from different parts of the metropolitan region.

The participating healthcare professional wrote 12 prescriptions, with 2 refills allowed per prescription, so that 3 prescriptions could be filled at each of the 12 pharmacies (36 total fills) for compounded 2% diltiazem cream. The prescriptions were filled at each of the 12 pharmacies during May 2012 and June 2012, at intervals of 1-2 wk in between refills. As soon as any prescription was filled by a retail pharmacy, it was collected and shipped at ambient temperature in a prepared bubble-wrap mailer to DermPathe Pharmaceuticals (Branchberg, NJ, United States), the laboratory engaged for the HPLC analysis. The compounded formulations of topical 2% diltiazem were provided by the retail pharmacies as either jars or tubes.

Upon receipt of each package, DermPathe logged the time and date and stored the compounded formulation at ambient temperature. HPLC analysis was conducted

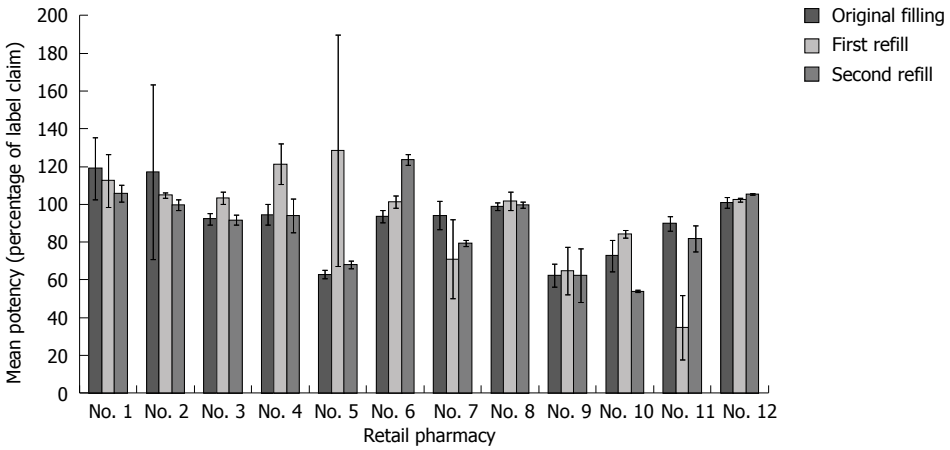

Figure 1 Mean potency of diltiazem content in each of the 3 samples from each of the 12 different retail pharmacies (mean ± SD).

in accordance with the DermPathe-validated method for diltiazem in cream, ointment, and gel formulations. The analysis included an assessment of potency (percentage of label claim) and content uniformity for each of the compounded formulations. For the analysis of each formulation, 8 different samples of the topical diltiazem, each approximately 1 g in weight, were taken from prespecified locations within the compounded formulation container. If the compounded formulation was provided in a jar, 4 samples were taken from the top of the jar: 1 at the top front, 1 at the top center, 1 at the top right back corner, and 1 at the top left back corner. In the middle layer of the jar, 1 sample was taken from the center and 1 from the middle right corner. At the bottom of the jar, 1 sample was taken from the center and 1 from the bottom left corner. If the compounded formulation was provided in a tube, the tube was sectioned horizontally and opened up. Then 2 samples were drawn from the top left and top right of the tube, 2 more from the middle left and middle right of the tube, 2 from the bottom left and bottom right of the tube, and the final 2 randomly on the left and on the right between the middle and the bottom of the tube.

Each sample was transferred to a 100 mL volumetric flask, to which methanol was added to fill approximately 80% of the flask volume. The solution was sonicated for 1 h, and the flask was then filled to volume with methanol and mixed thoroughly by shaking. Filtered through a 0.45-micron filter, the solution was then transferred into HPLC vials for analysis using a Waters 2695 HPLC system with a 2487 dual wavelength detector (Waters Corporation, Milford, MA, United States). A Luna C8 150 mm × 4.6 mm, 5 mm column with a C8 Security Guard column (Phenomenex, Torrance, CA, United States) was used as the stationary phase. The mobile phase consisted of an acetate buffer, acetonitrile, and methanol, in a 50:25:25 ratio. The acetate buffer contained 8.2 g of anhydrous sodium acetate and 1.16 g of *D*-10-camphorsulphonic acid in 1000 mL of water, with pH adjusted to 6.2 with 1 mol/L sodium hydroxide. The flow rate was set at 2 mL per minute, the detector wavelength was set at 240 nm, and the column temperature was set to 30 ℃.

Statistical analysis

Descriptive statistics were used in this study. For categorical variables, frequencies and percentages are reported. For continuous variables, the number of observations, mean ± SD, and relative standard deviation are reported. Statistical analyses were performed using Excel 2010 (Microsoft, Redmond, WA, United States).

RESULTS

Thirty-six prescriptions for compounded topical 2% diltiazem were written, filled, and shipped to DermPathe for analysis. Of these preparations, 30 were packaged in jars and 6 were packaged as tubes (only 2 of the 12 retail pharmacies used tubes for packing). One of the 12 retail pharmacies failed to label each of the 3 filled prescriptions of topical 2% diltiazem with the drug name; the label on each of the 3 formulations from this pharmacy simply read "compound."

The prescriptions were specifically for cream formulations. Five of the 12 pharmacies compounded 2% diltiazem as a cream, using lipoderm, but 6 of the 12 pharmacies compounded 2% diltiazem as an ointment, using petrolatum. For 1 pharmacy, the same that had the inadequate labeling, the dosage form was unknown.

The preparations were analyzed by DermPathe within 10 d of receipt. Prior to being analyzed, the products were stored at room temperature. In the published directions for preparing topical 2% diltiazem, the shelf life of the preparation is given as 30 d when stored at room temperature[11]. At the time of HPLC analysis, there were no visible signs of product degradation in any of the jars or tubes.

Potency results

Of the 36 prescriptions, 18 (50.0%) were misbranded for potency according to the USP standard. Five (13.9%) of the prescriptions were suprapotent (that is, the measured drug activity was > 115% of label claim) (Figure 1). The suprapotent prescriptions ranged in potency from 117.2% to 128.5% of label claim. No retail pharmacy produced more than 1 suprapotent formulation. Thirteen

Table 1 Potency at prespecified locations of compounded preparations of topical 2% diltiazem that lacked content uniformity

	Percentage of label claim (prescription number, type of packaging)												
Sample location[1]	1, jar	2, jar	4, jar	10, jar	11, jar	14, jar	19, jar	20, jar	25, jar	26, jar	27, jar	28, jar	32, tube	33, tube
Location 1	111.9%	110.8%	102.9%	97.5%	126.0%	83.3%	100.2%	71.4%	73.0%	39.9%	94.8%	83.8%	44.3%	67.4%
Location 2	108.7%	114.2%	95.7%	98.0%	117.8%	73.1%	99.3%	20.9%	58.7%	74.2%	68.9%	66.5%	15.1%	83.4%
Location 3	142.1%	112.0%	100.6%	101.1%	144.9%	93.4%	91.2%	82.3%	67.3%	52.1%	63.4%	81.1%	16.8%	80.7%
Location 4	149.1%	145.5%	99.8%	100.9%	117.1%	72.4%	99.9%	81.8%	64.9%	76.1%	57.7%	82.9%	33.8%	87.9%
Location 5	110.6%	106.5%	109.0%	92.9%	107.4%	112.4%	95.9%	69.2%	63.2%	67.9%	54.3%	71.5%	67.7%	81.5%
Location 6	106.7%	99.9%	97.1%	90.9%	116.1%	161.1%	97.3%	81.3%	59.5%	65.8%	51.5%	65.4%	29.4%	79.3%
Location 7	110.8%	103.8%	100.8%	85.9%	121.2%	211.2%	77.8%	79.9%	55.2%	72.0%	56.1%	65.6%	42.0%	83.0%
Location 8	111.7%	107.8%	231.7%	89.2%	120.6%	220.8%	91.0%	82.1%	57.6%	70.6%	52.8%	65.6%	29.4%	90.7%
Mean	119.0%	112.6%	117.2%	94.6%	121.4%	128.5%	94.1%	71.1%	62.4%	64.8%	62.4%	72.8%	34.8%	81.7%
Relative SD	14.0%	12.5%	39.6%	6.0%	9.0%	46.9%	8.0%	29.4%	9.4%	19.2%	22.9%	11.5%	48.5%	8.5%

[1]Jars: 1: Top front; 2: Top left back corner; 3: Top right back corner; 4: Top center; 5: Center of jar; 6: Middle right corner; 7: Bottom left corner; 8: Bottom center. Tubes: 1: Top left; 2: Top right; 3: Middle left; 4: Middle right; 5: Random left; 6: Random right; 7: Bottom left; 8: Bottom right.

(36.1%) of the prescriptions were subpotent (that is, the measured drug activity was < 90% of label claim) (Figure 1). The subpotent prescriptions ranged in potency from 34.8% to 89.8% of label claim. Only 3 of the 12 pharmacies compounded each of the 3 prescriptions they filled without misbranding potency. For 3 of the 12 pharmacies, all 3 of the filled prescriptions were subpotent.

Content uniformity results

Of the 36 preparations, 14 (38.9%) lacked content uniformity according to the USP requirement of 90% to 110% potency and < 6% relative standard deviation[12]. Table 1 shows the potency variations at the different sample locations of these 14 preparations. Of the 30 formulations packaged in jars, 12 (40%) lacked content uniformity; of the 6 formulations packaged in tubes, 2 (33.3%) lacked content uniformity. In some of the jars the potency varied by more than 100%. In batch 4, provided as a jar, the potency at the top center of the jar was 99.8% while the potency at the bottom center of the jar was 231.7%. In batch 14, also provided as a jar, the potency at the top center of the jar was 72.4% while the potency at the bottom of the jar was 220.8%. Batch 32, provided as a tube, was overall subpotent and also lacked content uniformity: at the middle left of the tube, potency was 16.8%; at the bottom left of the tube, potency was 42.0%.

Pharmacy performance

Nine of the 12 pharmacies failed USP potency or content-uniformity specifications for at least 1 of their 3 prescriptions. Three of the 12 pharmacies failed USP potency or content-uniformity specifications for all 3 of their prescriptions. When the potencies of the 3 time-separated prescriptions were averaged together for each of the 12 pharmacies, the mean potency was < 90% of label claim for 5 of the 12 pharmacies (Table 2).

DISCUSSION

In this HPLC analysis of 36 preparations of compounded topical 2% diltiazem from 12 retail pharmacies, half of the preparations did not meet USP specifications for potency and almost 40% of the preparations did not meet USP specifications for content uniformity. Of the 12 retail pharmacies, only three were able to fill all three of the time-separated prescriptions consistently within USP specifications.

When compounded preparations of topical 2% diltiazem fall outside USP specifications for potency, they are more likely to be subpotent (36.1% of the prescriptions) than suprapotent (13.9% of prescriptions). With more than a third of the prescriptions of compounded 2% diltiazem being subpotent, such prescriptions might not routinely relieve anal fissure pain to the extent or with the speed established in clinical trials of topical 2% diltiazem[1,4,14-16]. In one of those trials, Carapeti *et al*[1] compared different diltiazem gel concentrations (0.1%, 0.5%, 1%, 2%, 5%, and 10% weight per volume) and found a dose-dependent effect on maximum resting anal sphincter pressure (MRP), with the maximal effect (28% reduction compared with pretreatment, $P < 0.0001$) achieved with the 2% formulation. The MRP was not lowered as effectively with the 1% concentration, while concentrations higher than the 2% produced no additional effect.

The potency of the 5 suprapotent preparations of topical 2% diltiazem did not exceed 128.5% of label claim. However, owing to a lack of content uniformity in some preparations, especially when packaged in jars, compounded diltiazem could be more than twice as potent as the label claim in some sections of a container. The level of suprapotency in these sections, as high as 231.7% of label claim in a section of one jar, could put patients at potential risk for drug-related side effects. Because diltiazem is also used as a hypertensive agent, the largest risk associated with suprapotent topical 2% diltiazem might be dizziness or postural hypotension. In studies that have evaluated topical 2% diltiazem for treatment of anal fissure, the side effect profile has been mild, and the most frequent side effects have been headache or anal pruritus[5,17-20]. However, there have been reports of postural hypotension associated with the use of topical diltiazem[21].

Azarnoff *et al*[12] conducted a similar HPLC analysis

Table 2 Mean potency as percentage of label claim for 3 prescriptions of compounded topical 2% diltiazem filled by 12 retail pharmacies

Pharmacy No.	Label claim (mean ± SD)	Relative SD
1	95.9% ± 9.8%	8.7
2	107.3% ± 16.5%	15.4
3	95.9% ± 2.2%	2.2
4	103.3% ± 5.1%	4.9
5	86.5% ± 20.1%	23.2
6	106.2% ± 1.8%	1.7
7	81.6% ± 6.5%	8.0
8	100.1% ± 2.0%	1.9
9	63.2% ± 3.8%	6.0
10	70.4% ± 3.2%	4.5
11	68.8% ± 6.5%	9.4
12	102.9% ± 0.8%	0.8

of compounded formulations of topical 0.3% nitroglycerin ointment for anal fissure. The investigators acquired 24 filled compounded prescriptions from 24 retail pharmacies across different geographic regions. They found that 7 (29.2%) of the 24 compounded formulations were subpotent and that 1 (4.8%) was suprapotent. Moreover, 5 (20.8%) of the 24 samples lacked content uniformity. In comparison, in the current study, in which 36 compounded preparations were acquired from 12 different pharmacies, 13 (36.11%) of the preparations were subpotent, 5 (13.9%) were suprapotent, and 14 (38.9%) lacked content uniformity. The relatively worse analytic performance of compounded topical 2% diltiazem formulations in the current study might be an artifact of study design. Another explanation might be that it is more difficult to prepare compounded diltiazem formulations rather than compounded nitroglycerin formulations in accordance with USP standards, although it is unclear why this might be the case.

In October 2012, an FDA report of an outbreak of fungal meningitis related to contaminated products produced by the New England Compounding Center (Framingham, MA, United States) brought to public awareness the practice and business of pharmacy compounding[22,23]. There are legitimate reasons for physicians to prescribe extemporaneously compounded drugs: for example, to provide patients with products like topical 2% diltiazem, currently recommended in the ASCR practice parameters for anal fissure but not approved by the FDA, or to create unique medications for specific patients, such as those who have documented allergies to certain drug ingredients or who require dosage forms different from those of FDA-approved drugs[24-26]. However, not only have both branded and generic drugs undergone FDA approval for safety and efficacy, they are also required by law to be produced under federal Good Manufacturing Practice (GMP) regulations in order to ensure their identity, quantity, potency, and purity[24]. In contrast, although extemporaneously compounded drugs might be formulated under professional pharmacy standards, these standards are inherently less rigorous than federal GMP quality standards. Because there is no federal surveillance of compounded drugs, the extent of quality and safety problems with compound drugs is unknown[24]. Clearly, a topical 2% diltiazem cream produced under GMP regulations is needed to avoid the large percentage of substandard compounded formulations of a drug specifically recommended by the practice parameters of a medical society.

There were limitations to this study. The sample size was small, and the collection of samples was restricted to a single major metropolitan area. None of the compounded formulations were analyzed for microbiologic content when received by the laboratory. Upon routine inspection of the formulations after 8 mo of refrigerated storage at 5 ℃, the laboratory discovered mold on some of them, and all samples were discarded. A future study of compounded formulations of topical 2% diltiazem will need to include analyses of microbiologic content, of possible drug degradation, and of the release of diltiazem from the formulations.

In conclusion, this study shows that when patients are prescribed topical 2% diltiazem cream for treatment of anal fissure in a major metropolitan region, they receive compounded formulations from retail pharmacies that are misbranded in respect to potency approximately 50% of the time and that lack content uniformity approximately 40% of the time. Because approximately one third of the compounded preparations were subpotent, patients treated with compounded formulations of topical 2% diltiazem might not receive the anticipated relief of pain associated with anal fissure.

COMMENTS

Background

The use of topical 2% diltiazem hydrochloride for treating anal fissures is supported by multiple clinical trials and is recommended in published medical society practice parameters. In countries where no commercially manufactured formulation of topical 2% diltiazem is available, prescriptions for the medication need to be extemporaneously compounded by retail pharmacies. Up to now, the quality of these compounded diltiazem formulations has not been evaluated.

Research frontiers

High-performance liquid chromatography analysis was undertaken with 36 preparations of compounded 2% topical diltiazem that were gathered from 12 different independent retail pharmacies in a major metropolitan area.

Innovations and breakthroughs

This is the first study to show that when patients are prescribed topical 2% diltiazem cream for treatment of anal fissure, they receive compounded formulations from retail pharmacies that are misbranded in respect to potency approximately 50% of the time and that lack content uniformity approximately 40% of the time. Because over one third of the compounded preparations were subpotent, patients treated with compounded formulations of topical 2% diltiazem might not receive the anticipated relief of anal-fissure pain.

Applications

By demonstrating that a sample of retail pharmacies compound a large percentage of substandard formulations of topical 2% diltiazem, the study underscores that this drug, recommended for use by medical society practice parameters, should be produced under Good Manufacturing Practice regulations to ensure its identity, quantity, potency, and purity.

Peer review

Local absorption of diltiazem depends on skin thickness and local inflammation. It is also proportional to the medication amount. This study is similar to a 2007 study concerning compounded formulations of nitroglycerin ointment for anal

fissure. The authors noticed a problem that is presumably unknown to gastroenterologists and surgeons: that compounded formulations of topical 2% diltiazem, recommended by the American Society of Colon and Rectal Surgeons for anal fissure therapy but not approved by the United States Food and Drug Administration, may be subpotent or suprapotent. Of 36 compounded preparations examined in the study, 38.9% lacked content uniformity, and 50% did not meet United States Pharmacopoeia specifications for potency.

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BRIEF ARTICLE

Colorectal cancer in patients under 50 years of age: A retrospective analysis of two institutions' experience

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Abstract

AIM: To investigate the epidemiological characteristics of colorectal cancer (CRC) in patients under 50 years of age across two institutions.

METHODS: Records of patients under age 50 years of age who had CRC surgery over a 16 year period were assessed at two institutions. The following documents where reviewed: admission notes, operative notes, and discharge summaries. The main study variables included: age, presenting symptoms, family history, tumor location, operation, stage/differentiation of disease, and post operative complications. Stage of disease was classified according to the American Joint Committee on Cancer TNM staging system: tumor depth; node status; and metastases.

RESULTS: CRC was found in 180 patients under age 50 years (87 females, 93 males; mean age 41.4 ± 6.2 years). Young patients accounted for 11.2% of cases during a 6 year period for which the full data set was available. Eight percent had a 1st degree and 12% a 2nd degree family CRC history. Almost all patients (94%) were symptomatic at diagnosis; common symptoms included: bleeding (59%), obstruction (9%), and abdominal/rectal pain (35%). Evaluation was often delayed and bleeding frequently attributed to hemorrhoids. Advanced stage CRC (Stage 3 or 4) was noted in 53% of patients. Most tumors were distal to the splenic flexure (77%) and 39% involved the rectum. Most patients (95%) had segmental resections; 6 patients had subtotal/total colectomy. Poorly differentiated tumors were noted in 12% and mucinous lesions in 19% of patients of which most had Stage 3 or 4 disease. Twenty-two patients (13%) developed recurrence and/or progression of disease to date. Three patients (ages 42, 42 and 49 years) went on to develop metachronous primary colon cancers within 3 to 4 years of their initial resection.

CONCLUSION: CRC was common in young patients with no family history. Young patients with symptoms merit a timely evaluation to avoid presentation with late stage CRC.

Key words: Colorectal cancer; Colorectal cancer screening; Sporadic colorectal cancer; Early-age onset colorectal cancer; Sigmoidoscopy

Core tip: Colorectal cancer (CRC) is rising among patients under age 50 years. In our study, the majority of patients did not have a family history of CRC and presented with advanced disease stages. In America, many physicians wrongly believe that CRC in patients under age 50 years is uncommon and mostly found in patients with a 1st degree family history of CRC. This misconception delays time to diagnosis, contributing to a more advanced disease stage on presentation. The authors hope, after reading this article, doctors will recommend timely and complete colon evaluations for

patients under age 50 years who present with rectal bleeding.

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INTRODUCTION

Colorectal cancer (CRC) remains a notable source of morbidity and mortality worldwide[1]. CRC is consistently the third most commonly diagnosed cancer in the United States. The American Cancer Society estimated that there would be 103170 new cases of colon and 40290 new cases of rectal cancer in 2012; in addition, 51690 deaths were predicted[2]. Although common, the overall incidence of CRC in the general population declined by 2.9% in men and 2.2% per year in women between 1998 and 2009[3]. An increase in the proportion of the population undergoing screening colonoscopy and the removal of benign precancerous polyps is thought to account for, at least, part of this decrease.

Patients with a first degree family history of CRC are advised to begin screening colonoscopy at age 40 or 10 years prior to the youngest age at which a family member with CRC was diagnosed. In addition, screening programs for CRC are now widely implemented for "average risk" patients, defined as those without a first degree family history of CRC or other risk factors, other than age. Universally, it is advised that screening begin at age 50 years for "average risk" patients. Asymptomatic patients under 50 years of age without a family history are excluded from almost all screening programs. Perhaps, in part, because of the age 50 years cut off many patients and doctors have a low index of suspicion for CRC in young patients without family history who present with bleeding or other symptoms. It is also the impression of many doctors and patients that the majority of young patients who develop CRC have a positive family history. Although less common than in older patients, sporadic CRC accounts for the majority of cases in patients under age 50 years.

The National Cancer Database Report on CRC noted that individuals under 50 years of age accounted for roughly 7% of all CRC in a 1990 study population of over 38000 patients[4]. As per the Surveillance Epidemiology and End Results (SEER) Program data from 1993 to 1997, patients younger than 55 accounted for roughly 12% of all CRC cases[5]. A recent study that examined data from the SEER Program cancer registries between 1992-2009 reported that the overall incidence of CRC per 100000 people (20-49 years age category) increased 1.6% and 1.7% per year in men and women, respectively, over this time period[3]. A review of SEER Program data from 2005-2009 provides more detailed information regarding CRC in younger patients; in patients under 20 years the incidence was about 0.1%, in those 20-34 years of age it was 1.1%, in the 35-44 years sub-group the incidence was 4.0% whereas in the 45-54 years group it was 13.4%[2]. The data suggests that the incidence in younger patients is increasing[3].

A number of reports regarding young patients with CRC have been published over the last few decades (Table 1); however, these reviews typically span many years, often include patients with familial syndromes and/or ulcerative colitis, and do not adequately comment on the relevant family history of the study patients[6-15]. This focused review regarding two institutions' experiences with patients under 50 years of age with CRC that came to surgery over a 16 year interval was conducted to determine: the volume of CRC operations for young patients, the proportion with a family history of CRC, the stage at presentation, the specific cancer location, and the presenting symptoms, if any.

MATERIALS AND METHODS

Patient population

Hospital records of patients under the age of 50 years who underwent CRC operations between July 1996 and May 2012 at two institutions were reviewed (New York Presbyterian Hospital, Columbia University Campus and St. Luke's Roosevelt Hospital, NY, United States). Specifically , the following documents where reviewed; admission notes, operative notes, discharge summaries, endoscopy records, and pathology reports. A subset of this data was also obtained from an IRB approved prospective data base of patients undergoing colorectal resection maintained by the senior author from 2006 to June 30, 2009 at New York Presbyterian Hospital and from July 1, 2009 until May 2012 at St. Luke's Roosevelt Hospital. This prospective database also provided the information regarding CRC patients over 50 years of age (n = 392) used in this study. Additional information for this retrospective review was obtained from office charts and from telephone interviews.

Study endpoints

The main study variables included: demographics, presenting symptoms leading to diagnosis, family history of CRC, tumor location, type of surgical resection, stage and differentiation of disease, and post operative complications. Patients with inflammatory bowel disease or known polyposis syndromes such as familial adenomatous polyposis syndrome, Gardner's syndrome and the like were excluded from the study.

Disease stage was reported according to the TNM Classification System used by the American Joint Committee on Cancer[16,17]. "T" refers to the size or direct extent of invasion of the primary tumor; "N" refers to the degree of spread to regional lymph nodes, if any; and "M"

Table 1 Comparison of previously published reports of young patients with colorectal cancer

Ref.	Patients with CRC	Interval (yr)	Patient age (yr)	With family history of CRC (%)[1]
Recalde *et al*[6]	40	19	< 36	NR
Sanfelippo *et al*[7]	118	12	< 40	NR
Simstein *et al*[8]	41	15	< 40	4 (10)
Safford *et al*[9]	120[3]	33	< 41	6 (5)
Pitluk *et al*[10]	31	10	< 40	1 (3)
Behbehani *et al*[11]	47[3]	11	< 40	NR
Adloff *et al*[12]	32	7	< 40	NR
Domergue *et al*[13]	78[2]	18	< 41	NR
Palmer *et al*[14]	105[2]	12	< 40	NR
Fante *et al*[15]	90[2]	9	< 51	18 (20)
Present series	180	16	< 50	20 (12)[3]

[1]Degree of relation not otherwise specified; [2]Series includes patients with familial adenomatous polyposis and/or ulcerative colitis; [3]Patients with first degree relatives with colorectal cancer. NR: Not reported; CRC: Colorectal cancer.

Table 2 Patients' presenting signs and symptoms of colorectal cancer *n* (%)

Clinical presentation	Patients
Rectal bleeding	99 (57)
Anemia	19 (11)
Abdominal pain	54 (31)
Rectal pain	7 (4)
Change in bowel habits	37 (21)
Weight loss	20 (11)
Bowel obstruction	16 (9)
Perforation	5 (3)
Perforated diverticulitis	1 (0.6)
Screening	5 (3)
Unknown	7 (4)

refers to the presence of distant metastases.

Statistical analysis

Statistical methods for comparing stage and tumor distribution between the under age 50 years and the 50 and over years groups included a 2-proportion *Z* test.

RESULTS

Patient demographics

One hundred eighty patients under 50 years of age (87 females, 93 males; range 17-49 years; mean 41.4 ± 6.2 years) underwent a CRC operation between July 1996 and May 2012 at the two institutions. In regards to the total number of patients (regardless of age) that underwent a CRC operation, complete data is only available for the period between July 2006 and May 2012; during this time period 437 CRC operations on adults were carried out of which 49 (11.2%) involved patients less than 50 years of age. When the total population of 180 patients under age 50 is considered, the distribution of CRC within age categories is as follows: under age 30 years, 8 patients (4%); age 30-39 years, 46 patients (26%); age 40-49 years, 126 (70%). Of note, 30% of the patients were younger than 40 years of age. One hundred and seventy patients (94%) reported symptoms upon presentation (Table 2).

Table 3 Location of cancers *n* (%)

Anatomic location of cancer[2]	Cancers
Right colon[1]	31 (17.2)
Transverse colon	13 (7.2)
Descending colon	17 (9.4)
Descending and sigmoid colon junction	2 (1.1)
Sigmoid colon	29 (16.1)
Rectosigmoid colon	19 (10.6)
Rectum	71 (39.4)

[1]Right colon includes cecum, ascending, hepatic flexure; [2]Two patients had synchronous cancers.

Family history of CRC

Family history data was available for 167 patients; 13 patients (7.8%) did not know their family history. Regarding the 167 patients with family history data, 14 patients (8.4%) had a first degree family history of CRC, 20 patients (12.0%) had a second degree history, and 6 patients (3.6%) had both a first and second degree family history of CRC. Thus, a total of 12% of patients had, at least, a first degree family history of CRC. Seventy six percent were sporadic CRC cases. One patient (age 42 years) presented with synchronous primary cancers of the cecum and splenic flexure and had a family history notable for 3 first and 1 second degree relatives with CRC. The Amsterdam criteria for hereditary non-polyposis colorectal cancer (HNPCC) were met in this patient[14]. Unfortunately, none of the patients in this series were evaluated for gene mutations associated with HNPCC (*i.e.*, *hMLH1*, *hMSH2*, *etc.*)[18].

Distribution of tumor location and colorectal resection

The majority of tumors (77%) were located distal to the splenic flexure, with 39% involving the rectum (Table 3). Proximal (right and transverse colon) cancers were noted in only 24% of patients. In comparison, a cohort of 392 CRC patients 50 years of age and older who underwent an operation between 2006 and 2012 at the same institutions by the same surgeons, presented with more proximal tumors (age < 50 years, 24% *vs* age ≥ 50 years, 43%; $P < 0.0001$) and fewer rectal tumors (age < 50 years, 39% *vs* age ≥ 50 years, 27%; $P = 0.002$) using 2-proportion *Z*-test (Table 4).

A formal colorectal resection was done in the majority of patients (95%), a transanal excision of a rectal cancer in 1 patient, colocolonic bypass for unresectable Stage 4 disease in 1 patient, and proximal diversion in 6 patients with obstructing, locally invasive cancers (Table 5). Twenty three abdominoperineal resections and 3 low anterior resections with mucosectomy and subsequent coloanal anastomosis were performed. A Hartmann's procedure was done in 4 patients with sigmoid or rectal lesions. Two patients (ages 34 and 42 years) had synchronous primary colon cancers and underwent subtotal colectomy. Twenty-

Table 4 Colorectal cancer staging *n* (%)

Present series	Value	SEER[2]	Value
Age < 50 yr			
Stage 1[1]	37 (21)	Localized	30%
Stage 2	47 (26)	Regional	40%
Stage 3	70 (39)	Distant	27%
Stage 4	26 (14)	Unstaged	3%
Age ⩾ 50 yr			
Stage 1	88 (22)	Localized	38%
Stage 2	143 (36)	Regional	37%
Stage 3	135 (34)	Distant	19%
Stage 4	26 (7)	Unstaged	6%

[1]Two patients had Stage 0 disease and one patient had Tis disease following polypectomy; [2]Surveillance Epidemiology and End Results (SEER) 18, 2000-2009 stage distribution. Localized (confined to primary site), regional (spread to regional lymph nodes), distant (cancer has metastasized).

four distal resection patients were temporarily diverted (16% of all patients with anastomoses). Regarding the surgical methods used in the 172 patients who underwent bowel resection, the breakdown is as follows: laparoscopic-assisted, 78 patients (45.3%); hand-assisted or hybrid laparoscopic/open technique, 29 patients (16.9%); and open methods, 65 patients (37.8%).

Staging distribution

According to the TNM system for cancer staging by the American Joint Committee on Cancer[16,17], 37 patients (21%) had Stage 1 disease, 47 patients (26%) had Stage 2 disease, 70 patients (39%) had Stage 3 disease, and 26 patients (14%) had Stage 4 disease (Table 4). Three patients who underwent neoadjuvant chemoradiation for T3 rectal lesions based on preoperative endorectal ultrasound imaging had no residual disease on final pathology at the time of colorectal resection and were considered as Stage 2 lesions. Likewise, 3 patients who had sessile polyp cancers removed colonoscopically (invasion into submucosa noted on pathology) who underwent formal resection that revealed no residual cancer or involved lymph nodes on pathologic evaluation were classified as having Stage 1 cancers. Twenty patients with Stage 4 disease had known hepatic involvement and 3 patients had peritoneal carcinomatosis diagnosed at laparotomy.

Thirty-five patients (19%) underwent neoadjuvant chemoradiation, 3 patients (1.7%) underwent neoadjuvant chemotherapy alone, 10 patients (5.6%) underwent adjuvant chemoradiation, 56 patients (31%) underwent adjuvant chemotherapy, and 1 patient underwent adjuvant radiation alone for bony metastases. Of note, compared to the cohort of 392 CRC patients 50 years of age or older who underwent an operation between 2006 and 2012, patients under age 50 more often presented with Stages 3 and 4 disease (age < 50 years, 53% *vs* age ⩾ 50 years, 41%; $P = 0.003$ using 2-proportion Z-test) (Table 4).

Histopathological evaluation

Moderately or well differentiated cancers were noted in 124 patients (69%) whereas poorly differentiated cancers were found in 22 patients (12%). Of those with poorly differentiated histology, 67 percent presented with advanced Stage CRC (Stage 3 or 4). Thirty-four patients (19% of total) had mucinous adenocarcinomas of which 62% had advanced stage CRC.

Table 5 Type of colon resection *n* (%)

Operation	Patients	Laparoscopic
Right colectomy	34 (19.8)	17 (50)
Transverse colectomy	2 (1.2)	2 (100)
Left colectomy	20 (11.6)	10 (50)
Descending and sigmoid colectomy	1 (0.6)	1 (100)
Sigmoid colectomy	22 (12.8)	15 (7)
Rectosigmoidectomy	24 (13.9)	21 (88)
Low anterior resection	40 (23.2)	26 (65)
Abdominoperineal resection	23 (13.4)	11 (48)
Subtotal/total colectomy	6 (3.5)	4 (67)
Total resections	172 (95.0)	107 (62)

Postoperative complications and short-term outcomes

Regarding postoperative complications, there was 1 anastomotic leak and 4 intra abdominal/pelvic abscesses (reoperation in 1 patient, percutaneous drainage in 3 patients). Other postoperative complications included: ileus, 6 patients; small bowel obstruction, 6 patients (all required reoperation); wound infection, 7 patients; wound dehiscence, 2 patients (reoperation × 2); urinary retention, 3 patients; portal vein thrombosis, 1 patient; *C. difficile* colitis, 1 patient (treated with antibiotics); and incisional hernia, 1 patient (surgically repaired). There were no deaths perioperatively. Twenty-two patients (13%) developed recurrence and/or progression of disease to date. Three patients (ages 42, 42, and 49 years) went on to develop metachronous primary colon cancers within 3-4 years of their initial resection.

DISCUSSION

It is well established that the incidence of CRC increases significantly beyond the 5th decade of life and continues to rise thereafter with increasing age. More recent reviews have shown that the percentage of CRC patients under 50 years of age has increased to approximately 12 percent[5]. Our data corroborates these findings as 11.2% of CRC patients in our study were younger than age 50 years.

Many people, lay and physician alike, falsely believe that the majority of patients under 50 years of age who develop CRC have a significant family history and are genetically predisposed to developing CRC. Interestingly, only 12% of patients in our study had a first degree relative with CRC and only 1 patient (age 42 years) met the Amsterdam criteria for HNPCC based on family history. A literature search revealed several reports regarding young patients with CRC that showed family history data[6-15] similar to that of our study findings. In the general population of CRC patients (all ages), an estimated 15%-20% of patients have a family history of colorectal

neoplasia[19]. Therefore, regardless of age at diagnosis, the vast majority of patients with CRC have sporadic disease and are "average risk" patients without a family or personal history of colorectal neoplasm, inflammatory bowel disease, polyposis syndromes, or other risk factors.

Are CRC's in the under 50 population different from tumors that occur in older patients? In the absence of detailed genetic analyses of the tumors we must use clinical and basic pathologic data to address this question. The stage breakdown data may be helpful in this regard, although it is influenced by factors other than the tumors' biologic aggressiveness (*i.e.*, the timeliness of diagnosis). Similarly, the differentiation profile of the tumors in the younger and older CRC populations permits comparison of the 2 groups.

SEER stage distribution data from 2000-2009 for individuals with CRC under 50 years of age noted that 30% had localized disease (confined to primary site), 40% had regional disease (spread to mesenteric lymph nodes), and 27% had distant disease (metastatic) at the time of diagnosis; thus, 67% had Stage 3 or 4 disease. In contrast, in the over 50 age group, 39% of patients had localized disease, 37% had regional disease and 19% had distant disease at diagnosis; thus, 56% had Stage 3 or 4 disease[2]. Our study results, as well as other investigators[8,10,11,13], support the notion that advanced Stage (Stage 3 or 4) at presentation is more common among young patients compared to older patients (50 years and older). Taken together, the available data suggests that younger patients with CRC more often present with advanced disease when compared to the general population[8,10,11,13].

Regarding tumor differentiation, previous studies report a greater percentage of poorly differentiated (19%-49% of total) and mucinous tumors (9%-49%) in younger CRC populations[3,8,20,21], whereas, in the current study, only 12% of patients had poorly differentiated adenocarcinomas and 20% had mucinous histology. Data concerning the general CRC population from two previously published studies suggests that about 15% of colorectal adenocarcinomas are poorly differentiated and 17% demonstrate mucinous histology[22,23]. The relatively low percentage of poor prognosis histologies in our study may be related, in part, to the small number of HNPCC patients in the study population since HNPCC is associated with a higher incidence of poorly differentiated and mucin producing tumors. Regardless, the histology data does not provide an explanation for the high incidence of advanced stage tumors seen in the current study population. As mentioned earlier, there may be factors other than unfavorable histology and aggressive tumor biology contributing to the high rate of advanced stage tumors seen in younger CRC patients.

It has been suggested that delays in diagnosis may account, in part, for the advanced Stage at presentation noted in many CRC patients under 50 years of age[14,24]. Young symptomatic patients may delay presentation to a physician out of ignorance, fear, or denial. Furthermore, when confronted with young, average risk patients, clinicians may attribute their symptoms to any number of common benign anorectal disorders. The already mentioned fixation on 50 years as the age after which CRC occurs likely figures into the practioner's thinking as well. Consequently, a full colorectal evaluation may not be carried out for months to years following the onset of symptoms. For example, in the current study multiple patients reported a history of intermittent rectal bleeding and were treated for "hemorrhoids" for a year or longer before referral for diagnostic endoscopy. In one patient with an 18 mo history of diarrhea and occasional bleeding with mucus, a corresponding note from a gastroenterologist stated that the change in bowel habits likely represented irritable bowel syndrome with hemorrhoids. This patient ultimately underwent a colonoscopy and was found to have a sigmoid cancer. Another patient who reported postpartum rectal bleeding was told by her family practitioner that the cause was most likely hemorrhoids. This patient was eventually referred for a colonoscopy two years later after developing abdominal pain at which time she had a palpable rectal mass; she proved to have a Stage 3 lesion.

The ramifications of delayed diagnosis, specifically presentation with a more advanced CRC stage with its attendant increased mortality, justify, in the authors' opinion, prompt and complete large bowel evaluation in all patients under 50 years of age who present with suspicious colorectal symptoms in order to "rule out" an occult neoplasm. Signs and symptoms that may prompt such an evaluation include: bleeding, heme-positive stool, anemia, changes in stool caliber or bowel habits, and abdominal pain or persistent distension of unclear etiology.

Current screening and surveillance guidelines

There are currently no screening guidelines in place by the American Society of Colon and Rectal Surgeons (ASCRS) or the American Cancer Society (ACS) pertaining specifically to young patients who present with symptoms that could signify an underlying neoplasm. The ASCRS[25] does recommend, in general, that anyone "with symptoms or signs that suggest the presence of CRC or polyps who fall outside the domain of screening should be offered an appropriate diagnostic evaluation." However, it appears that in the United States too few practitioners are following this recommendation when confronted with young patients who report bleeding or other symptoms.

Questions regarding cancer surveillance and screening for patients and their families commonly arise when caring for young CRC patients. The following guidelines from the ASCRS and the ACS have evolved over the last 3 decades; although there are some minor differences they are quite similar. People with a first-degree relative (parent, sibling, or child) who has had CRC or adenomatous polyp(s) are advised to have screening colonoscopy starting at age 40 or ten years younger than the age, at diagnosis, of the youngest family member with CRC (whichever comes first) with repeated evaluation every

5 years. Individuals with two second degree relatives (grandparent, aunt, or uncle) with CRC or polyps are advised to be screened as average risk patients (see below), but beginning at age 40 years. Individuals with a single second or third degree relative (cousin, great-grandparent) with CRC or polyp(s) are advised to follow average risk screening guidelines. Individuals in HNPCC kindreds are advised to begin colonoscopic evaluation starting at 20-25 years of age or 10 years before the age of diagnosis in the youngest CRC patient in the immediate family, whichever comes first[25].

In average risk patients the ASCRS[25] recommends that routine screening commence at age 50 years. As per the ASCRS, acceptable screening strategies for average risk patients include: (1) flexible sigmoidoscopy every 5 years; (2) a double contrast barium enema every 5-10 years; or (3) a colonoscopy every 10 years. The ACS recommendations are almost identical to those of the ASCRS except that they include virtual colonoscopy every 5 years as an acceptable screening option. In regards to average risk patients under age 50 years without family history, the ASCRS[25] advises that they commence annual digital rectal examination and fecal occult blood testing at age 40 years; the ACS makes no recommendations for this group.

Future directions

Is endoscopic screening indicated for asymptomatic patients under 50 years of age? The rising incidence of CRC in this group and the tendency towards advanced Stage at presentation would argue in favor of such programs. Yet, the cost of screening colonoscopy programs would be very high and given the current economic climate and the fact that the incidence of CRC is still considerably lower in this group (*vs* the over age 50 population) the initiation of such programs is highly unlikely. However, perhaps a case can be made for a single screening sigmoidoscopy at age 40. In the present series a screening sigmoidoscopy would most likely have revealed a significant number of the neoplasms. As per the ACS, sigmoidoscopy identifies 70%-80% of individuals with advanced lesions and is associated with a 60%-80% reduction in CRC mortality for the area of the colon within its reach. Furthermore, in a recent multi-center randomized trial a single screening sigmoidoscopy carried out between the ages of 55 and 64 reduced CRC incidence by 33% and mortality by 43%[26]. Sigmoidoscopy, although invasive, is a safe procedure with a very low rate of perforation that is well tolerated without sedation. Although the chances for successful initiation of a flexible sigmoidoscopy screening program for young patients are small, the authors believe that our current dismal record with young CRC patients justifies the effort.

Study limitations

The lack of genetic testing for HNPCC in this study population is a clear weakness of this study. Similarly, the lack of long term cancer recurrence and survival results is also a major shortcoming. Ideally, genetic categorization of the tumors *via* microarray would be done which would permit a detailed analysis and comparisons.

In conclusion, this review corroborates recent national data regarding the rising incidence of CRC in the under age 50 population and the fact that a greater percentage of younger patients present with Stages 3 and 4 disease when compared to the entire CRC population. The vast majority of the young patients in this study had sporadic CRC; only 12% had a first degree family history of CRC and only 1 patient met the clinical criteria for HNPCC. The histologic breakdown of the tumors was similar to that reported in the general population of CRC patients.

Although it is impossible to confirm, it is the impression of the authors that in many patients there was a delay, either by the patient, physician, or both, in carrying out the appropriate diagnostic evaluation. The widely held belief that CRC occurs in patients age 50 and older likely contributes to this mind set. Clearly, at the very least, young symptomatic patients with rectal bleeding, a change in bowel habits, or abdominal pain should be promptly evaluated, preferably with a full colonoscopy. Finally, the medical and surgical community need to consider the concept of some type of screening program for young patients, perhaps flexible sigmoidoscopy, beginning at age 40 years. The goal is to diagnose CRC in this population at an earlier stage so that the recurrence rates and mortality can be reduced. The medical community and the public must be made aware of the fact that CRC occurs in patients under age 50 years with regularity and that thorough evaluation is indicated for symptomatic patients regardless of their age.

COMMENTS

Background

While the incidence of colorectal cancer (CRC) is declining in the overall population, it is on the rise among individuals under 50 years of age. Many patients and practioners, alike, believe most cases of early-age onset CRC are attributed to a family history of CRC; however, a growing number of small studies now show that the majority of these young patients have no family history.

Research frontiers

A growing number of small studies are being published that examine the rising incidence of CRC among patients under age 50 years. It is becoming increasingly evident that the majority of these patients do not have a family history of CRC; however, it is unclear what predisposition these patients have to develop CRC at an earlier age. The research hotspot may be to perform genetic categorization of these tumors *via* microarray analysis to determine how they differ from sporadic CRC in older patients.

Innovations and breakthroughs

Previous studies in the literature that have examined the incidence of early-age onset CRC often include patients with hereditary, familial, and sporadic CRC's (see below). In the study, the authors excluded patients with a known genetic CRC predisposition or history of inflammatory bowel disease. The vast majority of patients under age 50 years in the study had no reported family history of CRC and did not meet criteria by family history for hereditary nonpolyposis colorecal cancer (HNPCC). Therefore, the authors believe the majority of patients in the study had early-age onset "sporadic" CRC similar to the general population.

Applications

The study corroborates the findings of several other studies evaluating early-age onset CRC in that the majority of patients have no family history of CRC and most tumors were found in the distal colon and rectum. This contradicts the

believe of many practioners that early-age onset CRC's are more often located in the proximal colon and are attributable to inherited predispositions (*i.e.*, hereditary non-polyposis colorectal cancer). The next logical step is to investigate what triggers the development of a "sporadic" CRC at a younger age of onset among some individuals.

Terminology

Familial CRC is defined as CRC that presents 10-20 years earlier than the general population with no clear inheritance pattern. It is presumed that lower-penetrance susceptibility genes may play a role. Hereditary CRC accounts for approximately 5%-10% of patients diagnosed with CRC. Examples include familial adenomatous polyposis syndrome and HNPCC (Lynch Syndrome), which show autosomal dominant inherited germline mutations. Tumors are often located in the proximal colon and can be synchronous or metachronous in nature. Sporadic CRC occurs in patients without identifiable genetic predispositions or a reported family history of CRC.

Peer review

This is a descriptive study in which the authors investigate the incidence of early-age onset CRC among two institutions. The results corroborate a growing body of literature that now suggests that most CRC's, regardless of age of onset, occur without a particular genetic or familial predisposition.

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BRIEF ARTICLE

Cardiovascular disease risk factor profiles in children with celiac disease on gluten-free diets

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Abstract

AIM: To describe the cardiovascular disease (CVD) risk factors in a population of children with celiac disease (CD) on a gluten-free diet (GFD).

METHODS: This cross-sectional multicenter study was performed at Schneider Children's Medical Center of Israel (Petach Tiqva, Israel), and San Paolo Hospital (Milan, Italy). We enrolled 114 CD children in serologic remission, who were on a GFD for at least one year. At enrollment, anthropometric measurements, blood lipids and glucose were assessed, and compared to values at diagnosis. The homeostasis model assessment-estimated insulin resistance was calculated as a measure of insulin resistance.

RESULTS: Three or more concomitant CVD risk factors [body mass index, waist circumference, low density lipoprotein (LDL) cholesterol, triglycerides, blood pressure and insulin resistance] were identified in 14% of CD subjects on a GFD. The most common CVD risk factors were high fasting triglycerides (34.8%), elevated blood pressure (29.4%), and high concentrations of calculated LDL cholesterol (24.1%). On a GFD, four children (3.5%) had insulin resistance. Fasting insulin and HOMA-IR were significantly higher in the Italian cohort compared to the Israeli cohort ($P < 0.001$). Children on a GFD had an increased prevalence of borderline LDL cholesterol (24%) when compared to values (10%) at diagnosis ($P = 0.090$). Trends towards increases in overweight (from 8.8% to 11.5%) and obesity (from 5.3% to 8.8%) were seen on a GFD.

CONCLUSION: This report of insulin resistance and CVD risk factors in celiac children highlights the importance of CVD screening, and the need for dietary counseling targeting CVD prevention.

Key words: Celiac disease; Cardiovascular disease risk factors; Gluten-free diet; Insulin resistance; Children; Hyperlipidemia; Cholesterol

Core tip: In our study we demonstrate a relatively high proportion of children with celiac disease (CD) adherent to a gluten-free diet (GFD) with one or more cardiovascular disease (CVD) risk factors. Furthermore, this is the first report of insulin resistance in celiac patients either in adults or children. These findings suggest that screening for CVD risk factors in celiac children both at diagnosis and during follow-up is important. Furthermore, dietary counseling over time, targeting obesity and CVD risk factors in addition to monitoring adherence to a GFD in children and adolescents diagnosed with CD, may be warranted.

Norsa L, Shamir R, Zevit N, Verduci E, Hartman C, Ghisleni D, Riva E, Giovannini M. Cardiovascular disease risk factor profiles in children with celiac disease on gluten-free diets. *World J Gastroenterol* 2013; 19(34): 5658-5664 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i34/5658.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i34.5658

INTRODUCTION

Celiac disease (CD) is a common gastrointestinal autoimmune disorder characterized by inflammation of the small bowel mucosa triggered and sustained by ingestion of gluten in genetically predisposed individuals[1]. The prevalence of CD worldwide ranges between 0.5% and 3% of the general population[2-4]. Although CD has traditionally been considered a malabsorptive disorder associated with diarrhea and weight loss, these symptoms are now seen less frequently[5]. Several recent studies have reported that only a minority of newly diagnosed patients were underweight. Instead, many patients, both children and adults were overweight or even obese[6,7].

A definitive diagnosis of CD is currently made using IgA anti-tissue transglutaminase (tTG) antibody screening followed, in most cases, by confirmatory biopsies of the small intestine with compatible histopathological findings[8]. Currently, the only treatment for CD is a strict, life-long gluten-free diet (GFD) which leads to rapid clinical improvement, especially in children. The normalization of serological tests usually occurs 6 to 12 mo after initiation of a GFD[2].

Deranged adiposity, blood lipid profile abnormalities and other risk factors for cardiovascular disease (CVD) in CD patients are still debated, and clear conclusions have yet to be reached[9]. Several studies have demonstrated that CVD risk factors, namely obesity, abnormal blood lipid profile, hypertension and insulin resistance have their roots in childhood and tend to track into adulthood[10-12]. The primary aim of this study was to describe CVD risk factors in a population of celiac children adherent to a GFD for at least one year, in two Mediterranean countries.

MATERIALS AND METHODS

This cross-sectional multicenter study was performed at Schneider Children's Medical Center of Israel (Petach Tiqva, Israel), and San Paolo Hospital (Milan, Italy) between June 2010 and December 2011.

The study population included individuals less than 18 years old, previously diagnosed with CD, without known co-morbidities, who were referred for follow-up to the pediatric gastroenterology clinics of the participating centers. Patients were included if they had a definitive diagnosis of CD, ascertained by both positive serology and confirmed by compatible duodenal biopsies, and who had been on a GFD for at least one year with complete normalization of their CD serology (tTG antibodies) at the time of enrollment.

At enrollment, all patients underwent physical examination including measurement of weight using a digital scale, and height using a stadiometer. Measurement of waist circumference was performed with a tape measured to the nearest 0.5 cm at the midpoint between the bottom of the rib cage and the top of the iliac crest. Blood pressure was measured in the sitting position using an appropriately sized cuff. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. To evaluate BMI values across different age and gender groups we used the BMI standard deviation score percentiles that were calculated according to the Center of Disease Control and Prevention growth charts of 2000[13]. Children with BMI values lower than the 5th percentile for age and gender were classified as "underweight", those in the 5th to 85th percentile were classified as "normal weight", those in the 85th and 95th percentile were classified as "overweight" and those greater than the 95th percentile were classified as "obese"[13]. Pre-hypertension was defined as an average systolic or diastolic blood pressure between the 90th and 95th percentile for sex, age, and height-percentile-specific, and hypertension if the values were above the 95th percentile[14]. The same pediatrician performed the Tanner stage of puberty.

After an overnight fast of at least 8 h, blood samples were drawn for fasting glucose and insulin, triglycerides, total cholesterol, high density lipoprotein (HDL) cholesterol and tTG antibodies. The samples were analyzed in local laboratories. tTG antibody levels were quantified by enzyme linked immunosorbent assay. Serum glucose level was measured by the enzymatic UV test method using an automated analyzer and total cholesterol, triglycerides, and HDL cholesterol concentrations were measured by an enzymatic colorimetric method on an automated analyzer. LDL cholesterol was calculated using the Fridewald equation: LDL cholesterol = total cholesterol - [HDL cholesterol + (triglyceride/5)]. Serum insulin concentrations were measured by an immunometric assay with the Immulite 2000 Analyzer. According to the American Academy of Pediatrics (AAP) criteria, borderline levels of cholesterol were defined by values between the 75th and 95th percentile of LDL cholesterol, while values greater than the 95th percentile were considered elevated[15]. Insulin resistance was estimated by the homeostatic model assessment (HOMA-IR), as follows: HOMA-IR = [fasting insulin (μU/mL) × fasting glucose (mmol/L)/22.5). Although the hyperinsulinemic euglycemic clamp is the only validated method to evaluate insulin sensitivity in the pediatric population, HOMA has been widely used to estimate insulin resistance in the screening of large populations of euglycemic children[16]. In this study, insulin resistance was defined as HOMA > 3.16 according to the most recent cut-off for the pediatric population[17]. Based on the Bogalosa Heart Study[11], we analyzed six risk factors for CVD. The CVD risk factors considered were BMI *Z*-scores greater than the 85th percentile, waist circumference over the 90th percentile[18], fasting LDL cholesterol or triglycerides higher than the 75th percentile,

Table 1 Descriptive data of the study populations at diagnosis (T0) and recruitment (T1)

Variable	Israel	Italy	*P*-value
n	70	44	
Sex (female)	71.40%	59.10%	0.176
Age at diagnosis (mo)	77.0 ± 43.5	68.7 ± 48.5	0.185
Duration of GFD (mo)	38.9 ± 30.4	69.7 ± 55.6	< 0.0001[1]
Weight Z-score			
T0	-0.405 ± 1.21	-0.931 ± 1.36	0.357
T1	-0.172 ± 1.25	-0.240 ± 1.19	0.881
Height Z-score			
T0	-0.397 ± 1.14	-0.682 ± 1.40	0.455
T1	-0.192 ± 1.78	-0.310 ± 1.02	0.227
BMI Z-score			
T0	-0.103 ± 1.1	-0.369 ± 1.0	0.489
T1	-0.025 ± 1.2	-0.162 ± 1.2	0.760

[1]Statistically significant. Values are mean ± SD or number of subjects (percentage). BMI: Body mass index; GFD: gluten-free diet.

systolic or diastolic blood pressure greater than the 90th percentile and the state of insulin resistance[19].

In addition, we retrieved all the available data on anthropometry, blood lipids and glucose profiles at the time of diagnosis of CD from patient files.

This study was approved by the institutional review boards at each of the participating centers. Signed informed consent was provided by a legal guardian of each participant.

Statistical analysis

Descriptive data are shown as mean ± SD or number of observations (percentage). Symmetry of distribution of the variables was tested by the Kolmogorov-Smirnov test ($P > 0.05$). Triglycerides were not symmetrically distributed and were therefore log-transformed for analysis. Comparisons between the groups for continuous variables were performed by the *t* test for unpaired data or the Wilcoxon-Mann-Whitney test as appropriate. The χ^2 test for unpaired discrete variables and the Wilcoxon-Mann-Whitney test for paired discrete variables were used in this study. Additionally, a multiple logistic regression analysis was performed to assess the independent association of the center with HOMA-IR, adjusted for age, gender, BMI *Z*-score, Tanner stage and duration of diet. All *P*-values less than 0.05 were considered to indicate statistical significance (two tailed test). The SPSS software, version 18.0 (SPSS Inc, Chicago, IL, United States) was used for the statistical analysis.

RESULTS

During the study period, 114 children with a mean age of 10.4 (± 4.1) years (70 from Israel and 44 from Italy) were enrolled. The two populations of children had comparable demographic data and anthropometry both at diagnosis and after at least 1 year of a GFD (Table 1). The only significant difference was a longer follow-up period in the Italian children. Complete fasting lipid profiles prior to initiation of the GFD were available for 52/114 children, 36/70 from Israel and 16/44 from Italy, and insulin levels were not available from CD diagnosis as prior to our study the screening of lipid and glucose profiles was not routinely performed in patients with suspected CD.

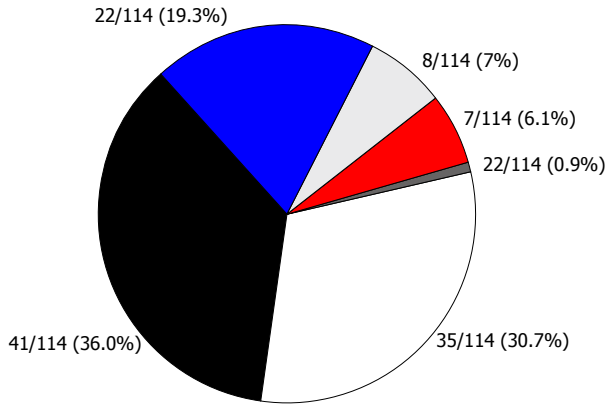

Figure 1 Risk factors for cardiovascular disease in pediatric patients with celiac disease in serological remission on gluten-free diets. White: 0 risk factor; Black: 1 risk factor; Bule: 2 risk factors; Light gray: 3 risk factors; Red: 4 risk factors; Dark gray: 5-6 risk factors. Risk factors sought included BMI *Z*-scores greater than the 85th percentile, waist circumference over the 90th percentile[18], fasting low density lipoprotein cholesterol or triglycerides higher than the 75th percentile, blood pressure systolic or diastolic greater than the 90th percentile and insulin resistance[19].

CVD risk factors

Overall, 14% of the cohort had 3 or more concomitant risk factors (Figure 1). Only 30.7% of the cohort did not have any risk factors (Figure 1). No significant difference was seen in the prevalence of CVD risk factors between the two countries in the cohort. The most common CVD risk factors were high fasting triglycerides (34.8%), elevated blood pressure (29.4%), and high concentrations of calculated LDL cholesterol (24.1%).

Anthropometry

We did not find any significant difference in the anthropometrics data between the Israeli and Italian CD children (Table 1). Anthropometrics in the whole cohort prior to and following the introduction of a GFD revealed significant increases in both height and weight *Z*-scores with an increase in BMI *Z*-scores which did not reach significance (Table 2). When scores were pooled into the CDC BMI categories, we found that both the prevalence of overweight and obesity increased from 8.8% and 5.3%, respectively, at the time of diagnosis to 11.4% and 8.8%, respectively, after the introduction of a GFD. This trend did not attain statistical significance ($P = 0.105$).

Lipid profile

There were no significant differences in the lipid profiles between the Israeli and Italian cohorts, except for higher levels of HDL cholesterol in the Italian patients. According to AAP criteria, 63% of the patients in our cohort had normal LDL cholesterol, 30% had borderline and

Table 2 Changes in height, weight, and body mass index at diagnosis (T0) and recruitment (T1) *n* (%)

Variable	T0	T1	*P*-value
Z-score height	-0.447 ± 1.1	-0.238 ± 1.1	0.001[1]
Z-score weight	-0.567 ± 1.3	-0.198 ± 1.2	< 0.001[1]
BMI Z-score	-0.207 ± 1.1	-0.078 ± 1.2	0.103
BMI categories[2]			
Underweight	11/114 (9.6)	12/114 (10.5)	
Normal weight	87/114 (76.3)	79/114 (69.3)	
Overweight	10/114 (8.8)	13/114 (11.4)	
Obese	6/114 (5.3)	10/114 (8.8)	0.105

[1]Statistically significant; [2]Classification[13]. Values are mean ± SD or number of subjects (percentage). BMI: Body mass index.

7% had hypercholesterolemia after at least one year of a GFD (Table 2). Although data on the lipid profile before CD diagnosis were available only in 50% of enrolled patients, we found significant increases in both total cholesterol and HDL cholesterol in patients on a GFD. The categorization of the LDL cholesterol values highlighted an increase in the prevalence of borderline cholesterol levels (from 9.6% to 23.1%), which did not reach statistical significance ($P = 0.090$).

Insulin resistance

The Italian children were found to have both higher fasting insulin and HOMA-IR levels while on a GFD when compared to the Israeli cohort (Table 3). Four patients (3.5%) were identified with frank insulin resistance, three from Italy, and one from Israel (Table 3). Two of these had normal weight and the remaining patients were overweight.

DISCUSSION

This cross-sectional study is the first to describe the profile of CVD risk factors in a cohort of children with CD in serologic remission on a GFD. Furthermore, this is the first report of insulin resistance in children with CD on a GFD.

Less than one third of our cohort did not have any CVD risk factors, while 14% had three or more risk factors. This finding suggests that CVD screening may be important in pediatric CD patients both at diagnosis and during follow-up. Studies have demonstrated that an earlier onset and greater number of CVD risk factors increase the chance of atheromatous plaque formation[10,11].

Our study design, which did not include a healthy control group, did not intend to determine whether children with CD have a higher risk than the general population for the development of CVD. Further prospective studies are needed to evaluate if changes in lifestyle and environment are responsible for a higher cardiovascular risk in celiac patients compared with the normal population. Nevertheless, although this study is limited by the lack of data prior to initiation of a GFD, it may suggest that the clinical and dietary follow-up should target adiposity, lipid profile and other CVD risk factors in addition to the common practice of dietary monitoring of adherence to a GFD.

Table 3 Lipidic, glycemic and insulinemic profile at enrollment in the two populations *n* (%)

Variable	Israel	Italy	*P*-value
Total cholesterol (mg/dL)	158.3 ± 27.6	162.5 ± 24.9	0.570
Cholesterol LDL (mg/dL)	95.4 ± 21	89.9 ± 22.3	0.116
Cholesterol HDL (mg/dL)	49.9 ± 10.6	59.4 ± 12.4	< 0.001[1]
Triglycerides (mg/dL)	71.1 ± 25.2	62.7 ± 20.9	0.055
LDL cholesterol classes[2]			
Normal	44/70 (62.9)	28/44 (63.6)	
Borderline	22/70 (31.4)	12/44 (27.3)	
Hypercholesterolemia	4/70 (5.7)	4/44 (9.1)	0.742
Glucose (mg/dL)	83.4 ± 6.9	80.3 ± 8.8	0.046[1,3]
Insulin μU/mL	3.3 ± 2.7	7.5 ± 4.3	< 0.001[1,3]
HOMA index	0.69 ± 0.6	1.55 ± 1.0	0.001[1,3]
Insulin resistance			
HOMA-IR < 3.16	69/70 (98.6)	41/44 (93.2)	
HOMA-IR > 3.16	1/70 (1.4)	3/44 (6.8)	0.108

[1]Statistically significant; [2]AAP classification[15]; [3]*P*-values are adjusted for age, gender, body mass index (BMI) Z-score, Tanner stage and duration of diet. Values are mean ± SD or number of subjects (percentage). HOMA-IR: Homeostasis model assessment-estimated insulin resistance; LDL: Low density lipoprotein.

The introduction of a GFD in CD patients increases the intestinal absorption of both macro and micro-nutrients. This leads to improved weight and height in celiac children presenting with malabsorption (weight loss, failure to thrive, poor weight gain)[20]. In our cohort, the majority of patients were of normal weight at the time of diagnosis and the percentage of overweight or obese patients was higher than those who were underweight. This drift in clinical presentation is concordant with previous reports[7] and may be attributed to increased awareness and early diagnosis. Alternatively, it may be explained by the radical change in diet and lifestyle in developed countries in recent decades, in line with the increasing prevalence of overweight and obesity in the general population. The increased prevalence of overweight and obesity after the introduction of a GFD in this study, although not significant ($P = 0.10$), may suggest the potential of a GFD to increase weight even in children presenting as normal or overweight at the time of CD diagnosis. The influence of a GFD on BMI remains unclear both in adults and children[9]. In adults, the debate is mainly based on two discordant theories. Dickey *et al*[21] demonstrated further weight gain in patients already overweight at the time of CD diagnosis after the introduction of a GFD, while Cheng *et al*[22] showed a positive effect of a GFD by demonstrating weight gain in previously underweight patients and weight loss in those previously overweight. Furthermore a recent study[23] recruiting a very large cohort of adult patients found that strict GFD adherence could increase the prevalence of overweight and obesity in CD patients. Contrasting studies have also recently appeared in the pediatric literature. Valletta *et al*[24] reported an increase in the fraction of overweight children following the introduction of a GFD, while Brambilla *et al*[25] demonstrated a beneficial effect of GFD on BMI in the

majority of CD children. Reilly *et al*[26] found a beneficial effect of GFD on the BMI of overweight celiac children. Our data, demonstrating that a GFD increases the prevalence of overweight and obesity in children with CD, is in agreement with studies reporting increased weight as a potential adverse effect of GFD.

The data concerning LDL cholesterol after at least one year of a GFD suggests an important role for cholesterol as a CVD risk factor in our cohort. In this study, LDL cholesterol was the third most prevalent CVD risk factor in celiac children on a GFD.

The increase in total and HDL cholesterol after GFD introduction in comparison to levels prior to initiation of a GFD (available from a subset of patients), is concordant with some studies which theorized that derangement of intestinal absorption, chylomicron production and lipoprotein metabolism may underlie the finding of lower levels of total and HDL cholesterol in untreated CD, which can revert to normal after treatment[27-30]. In contrast, we found that the rate of borderline LDL cholesterol concentrations more than doubled (from 9.6% to 23.1%) following adherence to a GFD. This may be the result of a tendency in adult and adolescent patients to consume gluten-free products with high fat contents[31-33] in order to compensate for the withdrawal of common gluten-containing carbohydrates from the diet.

Our data seem to suggest that although the increase in the rate of borderline LDL cholesterol could raise the cardiovascular risk, the concomitant increase in HDL may be cardioprotective, and thus future studies looking at surrogate markers of atherosclerosis are needed to determine whether a GFD is harmful in this regard.

Four children (3.5%) on a GFD had insulin resistance. As far as we are aware, the only studies reporting HOMA-IR in CD were performed in patients with concomitant insulin-dependent diabetes mellitus (IDDM) 1[34]. It is not known whether insulin resistance was present on CD diagnosis. As such, this is the first description of the presence of insulin resistance in CD children.

Due to the lack of insulin levels before CD diagnosis, we were unable to assess whether such insulin resistance is directly related to the introduction of a GFD. Previous publications have reported that available gluten-free products (*e.g.*, gluten-free bread, pasta, pizza *etc.*) have much higher glycemic indices than their gluten-containing equivalents, ingestion of which may lead to increased secretion of insulin[35-37]. Our findings, along with the previously mentioned change in the pattern of CD presentation, may suggest that future assessment of fasting glucose and insulin in children diagnosed with CD before and during the introduction of GFD should be performed. This is especially true in light of the role of insulin resistance as a CVD risk factor, and a predisposing condition for the development of type 2 diabetes[19]. The significantly higher fasting insulin levels and HOMA-IR in our Italian cohort may be explained by genetic and dietary differences between the two groups[37]. Our findings suggest that despite the classical consideration of CD as a malabsorptive condition, metabolic derangements, generally not attributed to this condition, should be actively sought even in patients who are non-obese. Although our data may hint to insulin resistance as a new complication of CD, a word of caution is due, as this study was performed on a cohort of CD patients and data is lacking in the literature regarding the prevalence of glucose intolerance in the healthy, non-overweight/obese children and adolescents.

This study has a number of limitations such as the relatively small number of patients, the cross-sectional design which did not allow for pre-GFD levels of all measured parameters, and the lack of familial history for CVD risk factors which may have further impacted our findings. However, despite these limitations, we have described the presence of insulin resistance in pediatric CD for the first time, and specifically addressed other CVD risk factors in the pediatric CD population on a GFD in serological remission.

Prior to initiation of the study, the relationship between CD and CVD risk factors was not clear, and therefore screening of lipid and glucose profiles was not routinely performed in patients with suspected CD. Additionally, the similarity in most findings between patients from two different countries, suggest that these findings are neither geographically nor ethnically specific. Prospective studies are needed to delineate the role of the GFD in the development of CVD risk factors in celiac children.

In conclusion, this cross-sectional study demonstrates a relatively high proportion of children with CD adherent to a GFD with one or more CVD risk factors including insulin resistance. These findings suggest the importance of screening for CVD risk factors in celiac children both at diagnosis and during follow-up. Furthermore, dietary counseling over time, targeting obesity and CVD risk factors in addition to monitoring adherence to a GFD in children and adolescents diagnosed with CD, may be warranted.

COMMENTS

Background

Celiac disease has traditionally been considered a malabsorptive disorder associated with diarrhea and weight loss, however, these symptoms are now seen less frequently. Several recent studies have reported that only a minority of newly diagnosed patients were underweight. Instead, many patients, both children and adults were overweight or even obese.

Research frontiers

Several studies have demonstrated that cardiovascular disease (CVD) risk factors, namely obesity, abnormal blood lipid profile, hypertension and insulin resistance have their roots in childhood and tend to track into adulthood.

Innovations and breakthroughs

Deranged adiposity, blood lipid profile and other risk factors for CVD in celiac disease (CD) are still debated, and clear conclusions have yet to be reached. The authors' study aimed to describe CVD risk factors in a population of celiac children adhering to a gluten-free diet (GFD) for at least one year, in two Mediterranean countries.

Applications

The authors' results suggest the importance of screening for CVD risk factors in celiac children both at diagnosis and during follow-up. Furthermore, they highlight that dietary counseling over time should target obesity and CVD risk factors in addition to monitoring adherence to a GFD in children and adolescents

diagnosed with CD.

Terminology

The homeostatic model assessment is a method used to quantify insulin resistance and beta-cell function. This model correlated well with estimates using the euglycemic clamp method (r = 0.88).

Peer review

The present study is an appreciable work in the sense that it explores a concept that was not given attention before. With changing lifestyle and environment are celiac children more exposed to cardiovascular risk factors than normal population it needs to be validated in further prospective studies. The results of study convince for metabolic screening in celiac disease children in follow up visits and hence initiate early intervention to prevent cardiovascular morbidity.

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BRIEF ARTICLE

5-ASA colonic mucosal concentrations resulting from different pharmaceutical formulations in ulcerative colitis

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Abstract

AIM: To compare the mucosal concentrations of 5-aminosalicylic acid (5-ASA) resulting from different pharmaceutical formulations and analyse the influence of inflammation on the mucosal concentrations.

METHODS: The study included 130 inflammatory bowel disease (IBD) patients receiving 5-ASA as pH-dependent-release formulations (73 patients), time-dependent-release formulations (11 patients), or pro-drugs (18 patients). In addition, 28 patients were receiving topical treatment (2-4 g/d) with pH-dependent-release formulations. Endoscopic biopsies were obtained from the sigmoid region during the colonoscopy. The 5-ASA concentrations (ng/mg) were measured in tissue homogenates using high-pressure liquid chromatography with electrochemical detection. The *t* test and Mann-Whitney test, when appropriate, were used for statistical analysis.

RESULTS: Patients receiving pH-dependent-release formulations showed significantly higher mucosal concentrations of 5-ASA (51.75 ± 5.72 ng/mg) compared with patients receiving pro-drugs (33.35 ± 5.78 ng/mg, P = 0.01) or time-dependent-release formulations (38.24 ± 5.53 ng/mg, P = 0.04). Patients with endoscopic remission had significantly higher mucosal concentrations of 5-ASA than patients with active disease (60.14 ± 7.95 ng/mg *vs* 35.66 ± 5.68 ng/mg, P = 0.02). Similar results were obtained when we compared patients with the histological appearance of remission and patients with active histological inflammation (67.53 ± 9.22 ng/mg *vs* 35.53 ± 5.63 ng/mg, P < 0.001). Significantly higher mucosal concentrations of 5-ASA were detected in patients treated with both oral and topical treatments in combination compared with patients who received oral treatment with pH-dependent-release formulations alone (72.33 ± 11.23 ng/mg *vs* 51.75 ± 5.72 ng/mg, P = 0.03).

CONCLUSION: IBD patients showed significant variability in mucosal 5-ASA concentrations depending on the type of formulation, and the highest mean concentration was achieved using pH-dependent-release formulations.

Key words: 5-aminosalicylic acid; Inflammatory bowel diseases; Mucosal concentration

Core tip: We report on the concentrations of 5-aminosalicylic acid in the colonic mucosa of ulcerative colitis patients. Significant variations in concentration were observed that were dependent on the type of pharmaceutical formulation and the presence of active disease. Combined oral and topical therapy yielded higher tissue mesalamine concentrations. These differences should

be taken into account in treatment strategies, especially in view of the fact that mesalamine can induce mucosal healing in ulcerative colitis.

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INTRODUCTION

Mesalamine[5-aminosalicylic acid (5-ASA)]-containing formulations represent the first-line therapy for the treatment of mild to moderate active ulcerative colitis and the prevention of recurrence[1]. When 5-ASA is administered and is absorbed by the colonic epithelium, *N*-acetyltransferase 1 metabolises a large amount of the 5-ASA to *N*-Ac-5-ASA, an inactive metabolite that is secreted back into the intestinal lumen and excreted in the faeces[2]. Sulphasalazine (Salazopyrin EN) is a pro-drug composed of sulphapyridine and 5-ASA connected by an azo-bond. Salazopyrin is metabolised to sulphapyridine and 5-ASA by the bacterial azoreductases of the intestinal microbiota. Sulphapyridine is excreted in the urine after most of it is absorbed from the colon, acetylated in the liver, and conjugated with glucuronic acid. The main action of sulphapyridine is to carry the 5-ASA moiety to the colon while preventing its proximal absorption. Absorption through the colon is necessary for the efficacy of 5-ASA[3]. Side effects, such as nausea, heartburn, headache, anaemia, skin rashes, reversible abnormalities of sperm number and morphology, and, rarely, hepatitis and nephritis, occur primarily due to high plasma sulphapyridine concentrations, which can generally be detected in patients taking higher doses of sulphasalazine or in genetically predisposed individuals (slow acetylators)[4]. Alternative preparations include modified-release formulations (which are supplied with pharmacological coatings that dissolve at a given pH or in a time-dependent manner) and pro-drugs. In pro-drugs such as sulphasalazine, an azo-bond links 5-ASA molecules to a carrier molecule. Similar compounds include olsalazine and balsalazide. Olsalazine was the first formulation, and it contains two 5-ASA molecules linked by an azo-bond. Approximately 12%-16% of patients being treated with olsalazine may suffer from secretory diarrhoea[5-7]. Balsalazide consists of 5-ASA linked *via* an azo-bond to an internal carrier (4-aminobenzoyl-β-alanine). This formulation is not systemically absorbed. Modified-release formulations include delayed-release formulations (which release 5-ASA along a pH gradient) and sustained-release formulations (which release 5-ASA over a specified time interval) that are targeted to release 5-ASA in the lower small intestine and right colon. The pH-sensitive acrylic resin coat of Eudragit dissolves when the luminal pH rises above a critical value. Pentasa is a sustained-release formulation that is gradually released based on a time-controlled mechanism. It consists of ethylcellulose-coated microgranules from which mesalazine is released into the small and large intestine. Its ethylcellulose coating is a semi-permeable membrane that dissolves when hydrated[8]. Combination therapy with oral and topical mesalazine administration can achieve higher mucosal concentrations than oral treatment alone[9]. Several *in vitro* studies have established a direct dose-effect relationship between 5-ASA and most of its immuno-inflammatory targets[10]. Furthermore, *in vivo* studies have demonstrated that there are inverse relationships between mucosal 5-ASA concentrations and the endoscopic and histological scores and mucosal levels of sIL-2R (a marker of mucosal inflammation). Higher drug mucosal concentrations lead to lower disease activity[11]. It follows that inadequate mucosal concentrations will result in inadequate disease management, particularly in patients with Crohn's disease and especially for the prevention of post-operative recurrence[12]. As a result, we can state that the therapeutic efficacy of 5-ASA is directly related to its mucosal concentration. Nevertheless, a large degree of individual variability in mucosal mesalamine concentrations exists, which is possibly due to differences in intestinal behaviour, dosage, route of administration, and the severity of the colonic inflammation[13-16].

In this study, we focused on different pharmaceutical formulations of 5-ASA.

MATERIALS AND METHODS

Patients and endoscopic procedures

The study included 130 consecutive ulcerative colitis patients (mean age 47.76 years, range 23-84 years; 81 men and 49 women) who were referred to the Department of Surgical, Oncological and Gastroenterological Sciences, Gastroenterology Unit on continuous oral 5-ASA treatment. The general characteristics of the patients are shown in Table 1. All of the patients were receiving treatment with oral 5-ASA three times per day in one of three different pharmaceutical formulations: pH-dependent delayed-release formulations (73 patients at a dose of 2.4 g daily; Asacol Giuliani-Bracco Italy, Pentacol Sofar Italy), mesalamine pro-drug (18 patients at a dose of 3 g daily; Salazopyrin EN, Pfizer, Italy), and time-dependent sustained-release formulations (11 patients at a dose of 3 g daily; Pentasa, Ferring, Italy). There were 28 patients who received both oral and topical (2-4 g/d by enema) pH-dependent-release formulations. The patients receiving combined treatment (mean age 46.5 years, range 23-79 years; 64.2% male) were comparable with respect to age and gender distribution to patients receiving oral therapy alone (mean age 47.24 years, range 25-84 years; 67.1% male). No concomitant immunological, renal, or hepatic disorders were reported by any of the patients. Moreover, none of the patients were taking steroids, immunosuppressive agents, antibiotics, H2-receptor antagonists, or proton pump inhibitors. Colonoscopy was performed for surveillance or to detect symptom re-exacerbation. Bowel

Table 1 Demographic and clinical characteristics of patients

Characteristics	pH-dependent delayed release (n = 73)	Pro-drugs (n = 18)	Time-dependent sustained release (n = 11)
Age (mean ± SE) (yr)	47.24 ± 1.61	51.38 ± 2.39	47.54 ± 4.97
Gender (M/F)	49/24	8/10	6/5
Extent of disease			
Proctosigmoiditis	22%	22%	0%
Left colitis	10%	11%	0%
Pancolitis	68%	67%	100%
Age at diagnosis (mean ± SE)	34.88 ± 1.61	32.72 ± 2.30	31.09 ± 3.30
Duration of disease (yr)	11.56 ± 0.81	17.22 ± 4.21	15.00 ± 2.62
Time since last 5-ASA administration (h)	21.43 ± 1.22	23.88 ± 4.21	20.63 ± 2.52

M: Male; F: Female; 5-ASA: 5-aminosalicylic acid.

cleansing was achieved using a polyethylene glycol oral solution, 3-5 L, on the day before the colonoscopy. After the patients provided their informed consent, the time at which they took their last pill/enema was recorded, and two biopsies were taken from the sigmoid region at 25 cm from the anal verge. The observation of inflammatory changes in the colonic mucosa on endoscopy was considered endoscopic activity following the Baron classification, while the absence of mucosal inflammatory changes was considered endoscopic remission[17,18]. The histological activity of the disease was examined according to a semi-quantitative score that took into consideration the extent of lymphocytic and polymorphonuclear leukocyte infiltration, mucus depletion, crypt distortion, the presence of crypt abscesses, and lymphoid follicle activation. Histological remission was defined as the absence of inflammatory changes in the mucosa[19].

Chemicals and reagents

Purified 5-ASA was obtained from Acros (NJ, United States). Purified water and methanol were used for the high-pressure liquid chromatography (HPLC) analysis. The use of these products was important to reduce background current and noise within the HPLC-electrochemical detection system.

A stock solution of 5-ASA was prepared at 1 mg/mL in 0.2 mol/L protocatechuic acid (PCA), 100 μmol/L ethylenediaminetetraacetic acid (EDTA), and 100 μmol/L sodium metabisulfite and stored at 4 ℃.

Tissue preparation

The specimens were homogenised in 0.2 mol/L PCA, 100 μmol/L EDTA, and 100 μmol/L sodium metabisulfite at 4 ℃ and then centrifuged (1800 *g*) for 10 min. The supernatants were collected and filtered with 0.2-μm cellulose acetate filters and stored at -80 ℃. A sample volume of 20 μL was used throughout this study.

Quantification of 5-ASA

A sensitive HPLC method capable of measuring the mucosal 5-ASA concentrations was used. Briefly, analyses were performed on a chromatographic apparatus (Alliance Waters, United States) that consisted of a model 2695 solvent-delivery system and an electrochemical detector, Coulochem (ESA, United States) Model 5100A, that was integrated with Empower Software (Waters, United States).

Separation of the analytes was achieved using a reversed-phase DHBA-250 column (5 μm, 250 × 3.0 mm), and the analytes were detected on a high-sensitivity analytical cell model 5011, with the oxidation potentials of electrodes 1 and 2 adjusted to +750 mV to oxidise the 5-ASA.

The mobile phase consisted of 50 mmol/L sodium acetate, 50 mmol/L sodium citrate, 8% methanol, and 2% 2-propanol. The pH of the mobile phase was adjusted to 2.5 with phosphoric acid after the addition of the organic modifiers. The mobile phase was passed through the system at 0.5 mL/min.

The standard curve for 5-ASA was linear in the selected range (r^2 = 0.99) with an inter-assay coefficient of variation < 4%.

Statistical analysis

The mucosal 5-ASA concentrations in patients being treated with any pharmaceutical 5-ASA formulation and in patients with different endoscopic and histological degrees of activity were compared using an unpaired Student's t test and the Mann-Whitney test where appropriate. A P value of 0.05 or less was consideredsignificant. The data are presented as the mean ± SE. The 5-ASA concentrations are expressed as ng/mg tissue. The statistical analyses were performed using the statistical software package SPSS for Windows, version 13.0 (SPSS, Chicago, IL, United States).

RESULTS

The demographic and clinical characteristics of the patients being treated with different pharmaceutical formulations were similar (Table 1). Figure 1 shows the distribution of the mucosal concentrations of mesalamine in the sigmoid region for the three groups studied. The mean mucosal mesalamine concentration was significantly higher in patients being treated with pH dependent-release formulations than in patients being treated with pro-drugs (51.75 ± 5.72 ng/mg *vs* 33.35 ± 5.78 ng/mg, P = 0.01). Similarly, the concentration of mesalamine was significantly higher in patients being treated with pH-dependent-release formulations than in patients being

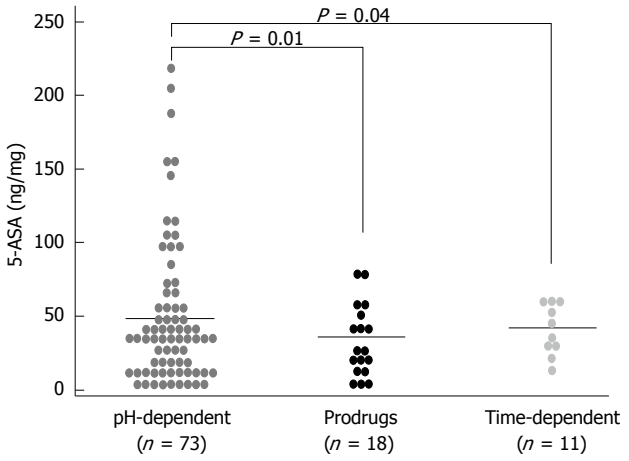

Figure 1 Distribution of 5-aminosalicylic acid mucosal concentrations in the sigmoid colon in patients receiving oral 5-aminosalicylic acid either pH-dependent release formulations, time-dependent release formulations or prodrugs. 5-ASA: 5-aminosalicylic acid.

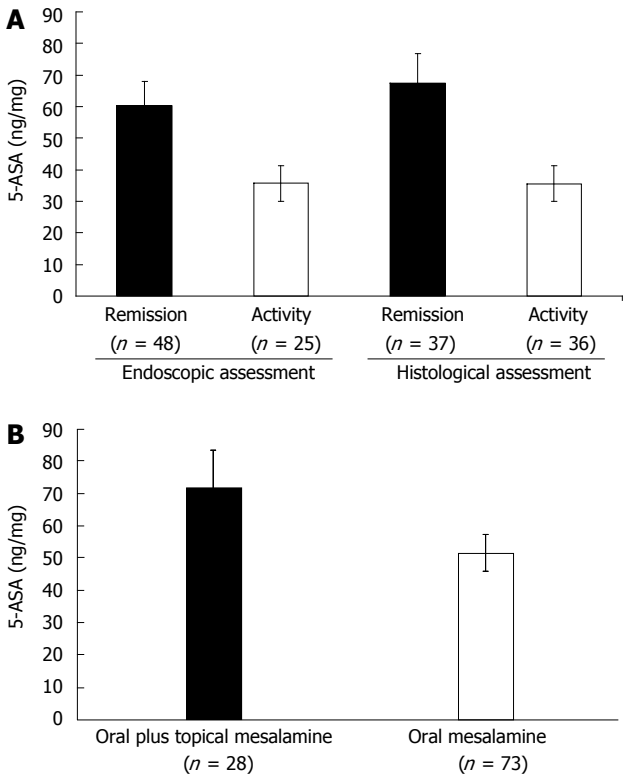

Figure 2 5-aminosalicylic acid mucosal concentrations in the sigmoid colon. A: In patients receiving oral 5-aminosalicylic acid (5-ASA) pH-dependent release formulations according to endoscopic and histological grading of disease; B: In patients receiving 5-ASA pH-dependent release formulations on oral plus topical treatment or oral therapy alone.

treated with time-dependent-release formulations (51.75 ± 5.72 ng/mg *vs* 38.24 ± 5.53 ng/mg, $P = 0.04$). Furthermore, the absolute mucosal 5-ASA concentrations were significantly higher in patients being treated with pH-dependent-release formulations; specifically, 28% of the patients were found to have mucosal 5-ASA concentrations above 70 ng/mg of tissue, which was the highest concentration achieved with any of the other formulations. Figure 2A shows the distribution of the mucosal mesalamine concentrations in patients receiving pH-dependent-release formulations according to endoscopic and histological activity or disease remission.

Twenty-five patients showed active disease in the sigmoid colon, while the remaining 48 patients presented an endoscopic appearance of remission or a normal assessment. The histological grade of the mucosal inflammation in the sigmoid colon was "active" in 36 patients, while the remaining 37 patients presented a "normal" histological assessment or the appearance of remission. Patients with a normal endoscopic assessment or an endoscopic appearance of remission showed significantly higher concentrations of mucosal 5-ASA than patients with active endoscopic inflammation (60.14 ± 7.95 ng/mg *vs* 35.66 ± 5.68 ng/mg, $P = 0.02$). Similarly, significant differences were found in the mucosal 5-ASA concentrations between patients with a normal histological assessment or the histological appearance of remission and patients with active histological inflammation (67.53 ± 9.22 ng/mg *vs* 35.53 ± 5.63 ng/mg, $P < 0.001$).

Twenty-eight patients received both pH-dependent mesalamine orally at a dose of 2.4 g/d and rectal mesalamine at a dose of 4 g/d. Figure 2B shows the distribution of mucosal 5-ASA concentrations in patients receiving combination treatment compared with those in patients receiving oral treatment alone. The mean mucosal 5-ASA concentration was significantly higher in patients receiving combined oral and topical treatment than in patients taking oral mesalamine only (72.33 ± 11.23 ng/mg *vs* 51.75 ± 5.72 ng/mg, $P = 0.03$).

DISCUSSION

IBD patients usually receive chronic treatment with 5-ASA to both control the active disease and reduce the frequency and severity of clinical relapses. Although this drug has been used for the last 50 years, the precise mechanism of action of 5-ASA, with the exception of its topical efficacy, is unknown[20]. However, its topical activity implies the necessity of its delivery to the inflamed tissue. Therefore, appropriate dosages and targeted delivery of the drug are needed. Many studies have demonstrated marked variability in 5-ASA metabolism and distribution following oral dosing[14,15]. Thus, the clinical course of the disease, which encompasses periods of prolonged remission and frequent episodes of relapse, could derive from variable availability of the drug. We know from several *in vitro* studies that there is a direct relationship between the 5-ASA concentration and its therapeutic efficacy[21,22]. Previous *in vivo* studies have reported that the therapeutic efficacy is dose-dependent. In fact, high mucosal mesalamine concentrations have been shown to be associated with endoscopic and histological scores of disease remission or mild activity rather than moderate or severe disease in ulcerative colitis. They have also been shown to be associated with a reduced risk of severe post-operative recurrences in Crohn's disease[11,12]. However, the appro-

priate mucosal concentration is unknown, and it is therefore not easy to provide guidance. There is wide inter-individual variability in mucosal concentrations, and the factors governing tissue drug concentrations are largely unknown because increased mucosal concentrations do not always derive from increased oral doses[23].

Adherence to therapy may be another important factor that influences mucosal concentrations. We did not test adherence specifically; however, samples were obtained only from patients who reported that they had taken their last pill within 24 h of endoscopy. Moreover, the 5-ASA concentration can be variable along the entire length of the colon. Oral administration of 5-ASA ensures a higher drug concentration in the right colon than in the rectum, where the amount often becomes negligible; however, the rectum is the preferential site of the disease and is almost invariably affected. Because the oral dose is not strictly related to the 5-ASA concentration, the tissue absorption, drug metabolism and excretion, and pharmaceutical variables, such as the route of administration and formulation type, need to be investigated. Several clinical studies have confirmed that the highest therapeutic efficacy is reached when patients are treated with oral and topical treatments[24,25]. Patients with active distal disease in whom oral treatment is frequently inadequate respond to mesalazine enemas[9]. Rectal formulations are also successful in maintaining remission in patients suffering from frequent relapses. According to Frieri *et al*[26], patients being treated with oral and topical treatments show similar mucosal mesalamine concentrations in the rectum and in the descending colon, while oral treatment alone results in a higher drug concentration in the descending colon than in the rectum. Indeed, we found higher mucosal concentrations in the sigmoid mucosa in patients receiving both topical and oral 5-ASA than in patients receiving oral mesalamine alone. It is possible that the level of adherence may have been higher in patients experiencing active disease who therefore were receiving combined therapy.

To date, few and discordant reports have investigated the relationship between the colonic mucosal concentration of 5-ASA and its different pharmaceutical formulations[27,28]. We demonstrated that the sigmoid mucosal concentration of 5-ASA was significantly higher in IBD patients receiving pH-dependent delayed-release formulations compared with patients receiving preparations dependent on bacterial degradation (pro-drugs). Similarly, the mucosal concentration of 5-ASA in the sigmoid mucosa of the pH-dependent delayed-release formulations group was higher than that of the time-dependent sustained-release formulations. Moreover, we found that the absolute mucosal 5-ASA concentrations were significantly higher in patients being treated with pH-dependent-release formulations than in patients being treated with pro-drugs. In fact, in 28% of the patients receiving pH-dependent-release formulations, the mucosal 5-ASA concentration was above 70 ng/mg of tissue, which was the highest value achieved from any of the formulations. Because of the dose-related anti-inflammatory effect of 5-ASA, we should expect the highest efficacy when the highest mucosal tissue concentration of 5-ASA is achieved. As reported by Hussain *et al*[23], rectal mucosal concentrations of aminosalicylates are lower during relapses. As previously demonstrated by Frieri *et al*[11], we confirmed that the colonic mucosal concentrations of 5-ASA were inversely related to disease activity as measured by both endoscopic and histological evaluation. Oedema occurring during the active phase of the disease may account for a possible dilution effect on the biopsy specimens. Alternatively, the faster rate of tissue renewal in the presence of inflammation could produce a wash-out effect on the drug and contribute to a reduced mucosal mesalamine concentration.

In conclusion, because we have reinforced the relationship between tissue mesalamine levels and disease activity, additional studies are needed to determine how to reach optimal mucosal concentrations of 5-ASA. We have demonstrated that different pharmaceutical preparations achieve different mucosal concentrations and that disease activity lowers the drug mucosal concentration. Higher dosages could therefore be justified during active disease to obtain better clinical results.

COMMENTS

Background

The use of mesalamine represents the first-line treatment strategy in patients with ulcerative colitis. Many formulations are available, and they are often used interchangeably because it is assumed that they are all equally effective.

Research frontiers

The mucosal concentration of 5-aminosalicylic acid (5-ASA) was measured in the colon of ulcerative colitis patients and at the anastomotic site of Crohn's disease patients in the post-operative setting. In this study, the authors demonstrated that tissue concentrations could be measured by high-pressure liquid chromatography and that the type of 5-ASA formulation influenced the mucosal concentration.

Innovations and breakthroughs

Mesalamine is poorly absorbed; therefore, blood monitoring is not helpful. The tissue concentration may represent a better tool for tailoring therapy in ulcerative colitis patients.

Applications

The response to treatment with mesalamine in ulcerative colitis patients can be optimised by administering the proper formulation of the drug at the right dose to the patient.

Peer review

The authors examined the colonic mucosal concentrations of mesalamine in patients with ulcerative colitis who were being treated with different formulations of mesalamine. The results are interesting and may guide clinicians in tailoring therapies to their patients.

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BRIEF ARTICLE

Eviendep® reduces number and size of duodenal polyps in familial adenomatous polyposis patients with ileal pouch-anal anastomosis

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Author contributions: Calabrese C initiated the study, coordinated the conduct of the whole study, performed the endoscopies; Calabrese C, Praticò C and Calafiore A prepared the draft of the manuscript; Calabrese C and Rizzello F re-evaluated all the endoscopy videos and photos in a blinded manner and scored the images separately; Coscia M, Gentilini L and Poggioli G performed the surgical procedures; Gionchetti P and Campieri M reviewed the manuscript; all authors approved the final version of the manuscript.

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Abstract

AIM: To evaluate if 3 mo oral supplementation with Eviendep® was able to reduce the number of duodenal polyps in familial adenomatous polyposis (FAP) patients with ileal pouch-anal anastomosis (IPAA).

METHODS: Eleven FAP patients with IPAA and duodenal polyps were enrolled. They underwent upper gastrointestinal (GI) endoscopy at the baseline and after 3 mo of treatment. Each patient received 5 mg Eviendep twice a day, at breakfast and dinner time, for 3 mo. Two endoscopists evaluated in a blinded manner the number and size of duodenal polyps. Upper GI endoscopies with biopsies were performed at the baseline (T0) with the assessment of the Spigelman score. Polyps > 10 mm were removed during endoscopy and at the end of the procedure a new Spigelman score was determined (T1). The procedure was repeated 3 mo after the baseline (T2). Four photograms were examined for each patient, at T1 and T2. The examined area was divided into 3 segments: duodenal bulb, second and third portion duodenum. Biopsy specimens were taken from all polyps > 10 mm and from all suspicious ones, defined by the presence of a central depression, irregular surface, or irregular vascular pattern. Histology was classified according to the updated Vienna criteria.

RESULTS: At baseline the mean number of duodenal detected polyps was 27.7 and mean sizes were 15.8 mm; the mean Spigelman score was 7.1. After polypectomy the mean number of duodenal detected polyps was 25.7 and mean sizes were 7.6 mm; the mean Spigelman score was 6.4. After 3 mo of Eviendep *bid*, all patients showed a reduction of number and size of duodenal polyps. The mean number of duodenal polyps was 8 (P = 0.021) and mean size was 4.4 mm; the mean Spigelman score was 6.6. Interrater agreement was measured. Lesions > 1 cm found a very good degree of concordance (kappa 0.851) and a good concordance was as well encountered for smaller lesions (kappa 0.641).

CONCLUSION: Our study demonstrated that short-term (90 d) supplementation with Eviendep® in FAP patients with IPAA and with recurrent adenomas in the duodenal mucosa, resulted effective in reducing polyps number of 32% and size of 51%.

Key words: Familial adenomatous polyposis; Ileal pouch-anal anastomosis; Duodenal polyps; Eviendep

Core tip: Our open study demonstrated for the first time that short-term (90 d) supplementation with Eviendep® in familial adenomatous polyposis patients with ileal pouch-anal anastomosis and with recurrent adenomas in the duodenal mucosa, resulted effective in reducing polyps number of 32% and size of 51%. Eviendep® was easy to manage and its daily use was well tolerated by the patients. Its safety was guaranteed by its composition. Each ingredient is blended into the composition in a lower dose than the one otherwise needed for the single component to similarly exert the desired effect, thus leading to synergistic and/or potentiating effect, with the added advantage of higher safety, even over long-term exposure.

Calabrese C, Praticò C, Calafiore A, Coscia M, Gentilini L, Poggioli G, Gionchetti P, Campieri M, Rizzello F. Eviendep® reduces number and size of duodenal polyps in familial adenomatous polyposis patients with ileal pouch-anal anastomosis. *World J Gastroenterol* 2013; 19(34): 5671-5677 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i34/5671.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i34.5671

INTRODUCTION

Familial adenomatous polyposis (FAP) is a disease with autosomal dominant inheritance. It is caused by an alteration of the *FAP* gene that is located on chromosome 5q21, affecting roughly 1 in 15000 live births in the Northern European population. FAP shares its phenotype with biallelic Homolog Gene Mutation Carriers, characterized by the early onset of hundreds to thousands of adenomas throughout the colon, with a nearly 100% progression to colorectal cancer by the age of 35-45 years in untreated subjects[1-3]. Patients with FAP have a cumulative lifetime risk of over 80% of developing duodenal adenomas, the precancerous lesions of duodenal adenocarcinoma. Consequently, these patients have a 4% lifetime risk of peri-ampullary or duodenal adenocarcinoma[4,5].

Early prophylactic colorectal surgery changed the prognosis of patients with FAP, and nowadays desmoids and peri-ampullary duodenal cancers are the most common causes of death in these patients[6,7].

Nearly 100% of FAP patients will develop duodenal adenomatosis[5-11] with an estimated lifetime risk of progression to duodenal carcinoma of 5%-10%[5-12]. The severity of duodenal adenomatosis is graded according to the Spigelman classification[10], which ranges from grade 0 to IV and is based on the number, size and histopathological features of the duodenal adenomas. Patients with advanced Spigelman stages are most at risk of developing duodenal carcinoma[5,10]. Current guidelines recommend frequent endoscopic surveillance in these patients, which improved the prognosis through earlier detection of duodenal malignancy[13].

A new recent line of intervention focuses on the role of the estrogen receptors (ERs) in intestinal carcinogenesis[14-16]. A pivotal role of ER-β has been suggested in preventing malignant transformation of colon epithelial cells in humans[16]. Data confirm the involvement of ERs-β in colorectal carcinogenesis and suggest a possible explanation for the protective effect of estrogens in cancer development[17-19]. They also provided further support of the role of vegetable-rich diets in the prevention of bowel cancer, thanks to their high content of phytoestrogens. Phytoestrogens include a variety of vegetable derived compounds with estrogen-like chemical structure and differential selectivity to the two ERs, ERα and ERβ. Particularly the dietary flavonolignan silymarin and the lignans have been reported to exert selective agonism to the ERβ the former, and preferential selectivity the latter. Since the promotion and progression of carcinogenesis are susceptible to nutritional interventions[5], the aim of this study was to evaluate if 3 mo oral supplementation with a patented blend of phytoestrogens and indigestible and insoluble fibres (Eviendep®, CM&D Pharma Limited, United Kingdom) was able to reduce the number of duodenal polyps in FAP patients with ileal pouch-anal anastomosis (IPAA).

MATERIALS AND METHODS

Population

This study was conducted in FAP patients with IPAA. The patients were ongoing the surveillance program at our department by screening upper gastrointestinal (GI) endoscopies for the follow-up of duodenal adenoma (polyp) recurrence and progression to adenocarcinoma. Cardiovascular diseases and inadequate organ function were study exclusion criteria. All patients gave their informed consent.

Endoscopic and histological procedures

Endoscopies were performed after an overnight fast; patients were prepared by a light sedation (*iv* midazolam coupled with 20 mg of scopolamine N-butyl bromide) and were examined with an upper GI endoscopy (Olympus GIF 165) until the third portion of the duodenum.

The severity of duodenal polyposis was classically assessed using the Spigelman classification[10]. This classification system describes five stages in duodenal polyposis development. Points are accumulated for number, size and histology of adenomatous polyps. Spigelman stage I (1-4 points) indicates mild disease, whereas stage Ⅲ-Ⅳ (> 6 points) implies severe duodenal polyposis. The traditional Spigelman classification classified adenomas into mild, moderate and severe dysplasia, whereas the updated classification distinguishes low- and high-grade dysplasia.

Upper GI endoscopies with biopsies were performed by the first operator (Calabrese C) at the baseline (T0) with the assessment of the Spigelman score. Polyps > 10 mm were removed during endoscopy and at the end

of the procedure a new Spigelman score was determined (T1). The procedure was repeated 3 mo after the baseline (T2), which also coincided with the 3 mo oral supplementation of Eviendep.

The first operator together with another experienced endoscopist (Rizzello F) re-evaluated all the endoscopy videos and photos. They evaluated images in a blinded manner and scored the images separately. Each expert first evaluated them individually and then in case of disagreement, a consensus was reached afterward by discussion.

Four photograms were examined for each patient, at T1 and T2. The examined area (photogram) was divided into 3 segments: duodenal bulb, second and third portion duodenum. For each segment the two operators were asked to assess the total number of polyps observed and their sizes by using an open biopsy forceps (8 mm).

Lastly, biopsy specimens were taken from all polyps > 10 mm and from all suspicious ones, defined by the presence of a central depression, irregular surface, or irregular vascular pattern.

Histologic samples were processed by using standard procedures and evaluated by gastroenterology specialized pathologists. Histology was classified according to the epithelium type (tubular, tubulovillous, or villous adenoma) and the degree of dysplasia (none, low grade, high grade, or cancer according to the updated Vienna criteria).

Treatment procedures

Eviendep® was chosen for its specifically high content of phytoestrogens and fibres. It comprises the selective ERβ-targeted flavonolignan silymarin (qualified for a 30% content in silibinin) and lignans (qualified for at least 40% of secoisolariciresinol diglucoside), in combination with non-starch, insoluble and indigestible fibres (qualified for or less than 5% lignin content). Each patient received 5 mg Eviendep twice a day, at breakfast and dinner time, for 3 mo.

Statistical analysis

Statistical significance was determined using Student's *t*-tests for paired and unpaired samples. Treatment results were compared by χ^2 test for comparison of proportion with a 95% confidence interval (CI). All statistical analyses were 2-tailed, and significance was accepted at a P value < 0.05. To test the reproducibility of these findings, interrater agreement was calculated with kappa analysis. A score of < 0.20 was considered poor, 0.21 to 0.40 fair, 0.41 to 0.60 moderate, 0.61 to 0.80 good, and 0.81 to 1.00 very good. We performed all the statistical analyses using a statistical software package (SPSS Inc, Chicago, IL, United States).

RESULTS

Eleven patients (M/F 5/6; mean age 40.7 years, SD ± 13.6 years) met the inclusion criteria and were enrolled; they were followed prospectively between November 2012 and January 2013 at our department outpatients' clinic. The mean age at colectomy was 23 ± 6.2 years and mean age of IPAA was 17.7 ± 8.5 years. Table 1 shows the demographic, clinical characteristics and polyps' histological features.

Table 1 Findings at baseline and patients characteristics in 11 jejunal polyposis patients

Patient No.	Age, yr (gender)	Age at colectomy (yr)	No. of duodenal polyps	Max size of duodenal polyps (mm)	Spigelman score
1	32 (F)	18	53	12	8
2	57 (M)	31	32	21	8
3	43 (F)	21	21	22	8
4	31 (F)	16	20	5	7
5	23 (M)	22	30	12	7
6	29 (M)	18	12	5	6
7	62 (M)	32	19	23	7
8	31 (F)	17	22	19	7
9	45 (M)	28	19	12	6
10	35 (F)	19	54	21	7
11	60 (F)	31	23	22	7

F: Female; M: Male.

At baseline (T0) the mean number of duodenal detected polyps was 27.7 ± 13.8 years (range 12-54 years) and mean sizes were 15.8 ± 6.8 mm (range 5-23 mm); the mean Spigelman score was 7.1 ± 0.7 (range 6-8). Histology confirmed tubular adenomatous tissue with low-grade dysplasia.

After polypectomy (T1) the mean number of duodenal detected polyps was 25.7 ± 13.4 (range 10-51) and mean sizes were 7.6 ± 1.9 mm (range 5-10 mm); the mean Spigelman score was 6.4 ± 0.5 (range 6-7). After 3 mo of Eviendep *bid* (T2), all patients showed a reduction of number and size of duodenal polyps. The mean number of duodenal polyps was 8 ± 6.2 (range 2-19) (χ^2 = 28.42, P = 0.021) and mean size was 4.4 ± 2.1 mm (range 2-9 mm); the mean Spigelman score was 6.6 ± 0.7 (range 4-6) (Table 2, Figure 1).

Interrater agreement was measured. Lesions > 1 cm found a very good degree of concordance (kappa value 0.851) and a good concordance was as well encountered for smaller lesions (kappa value 0.641).

All the eleven patients completed the study. Compliance was excellent; only one patient reported mild intestinal bloating, and the therapy was not discontinued.

DISCUSSION

Colonic surveillance programs and proctocolectomy with IPAA have improved the prognosis of patients with FAP. Current leading disease-related causes of death are desmoids tumours and duodenal adenocarcinomas. Duodenal cancer is nowadays the most important cause of death in FAP patients[13]. With respect to the duodenal manifestation of this disease, surveillance and prophylactic treatment strategies will hopefully further improve

Table 2 Findings at baseline after polypectomies and after 3 mo of treatment

Patient No.	T1			T2		
	Duodenal polyps (*n*)	Max size of duodenal polyps (mm)	Spigelman score	Duodenal polyps (*n*)	Max size of duodenal polyps (mm)	Spigelman score
1	50	10	7	19	4	5
2	30	8	7	10	8	6
3	20	9	6	4	9	5
4	18	5	6	2	3	4
5	29	8	7	19	4	5
6	10	5	6	4	2	4
7	18	8	6	10	4	5
8	19	10	6	4	4	4
9	17	8	6	2	3	4
10	51	8	7	10	4	5
11	21	5	7	4	4	4

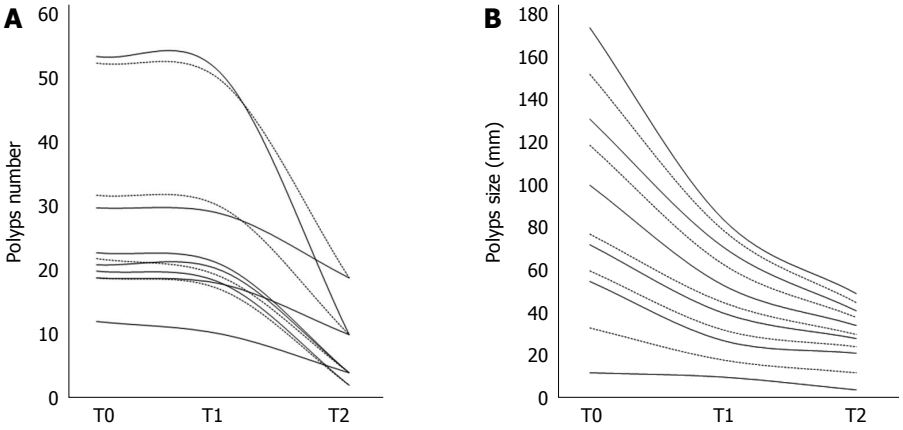

Figure 1 Changes in total polyps number and max size in all patients at baseline (T0), after polypectomy (T1) and after 3 mo of treatment (T2). A: Polyps number; B: Polyps size.

prognosis. The secondary chemoprevention of duodenal cancer identifies three different lines of intervention.

First chemopreventive strategy

Pharmacological intervention studies have been primarily focused on targeting the inflammatory pathway of cyclooxygenase-2 (COX-2), an enzyme with increased expression in experimental and human intestinal neoplasia. Most of these studies tested the efficacy of nonsteroidal anti-inflammatory drugs (NSAIDs) and acetylsalicylic acid on either adenoma regression or new polyps prevention[20]. Randomized placebo-controlled trials have demonstrated that the NSAIDs sulindac and the selective COX-2 inhibitors celecoxib and rofecoxib have chemopreventive efficacy in FAP, with a significative regression of polyps[21-23].

However, evaluations of the safety of the COX-2 inhibitors and NSAIDs showed an elevated risk of serious cardiac disorders, selected renal and hypertension events and a rebound effect on adenomas after the discontinuation of the treatments[24].

Second chemopreventive strategy

Dietary interventions are the second chemopreventive treatment line. The studies one these interventions are based on potential chemopreventive properties of several dietary components and on the evidence of epigenetic mutations of tumour suppressor genes in the intestinal mucosa after an unbalanced diet[25,26]. These studies forecasted an increase in servings/day of either fruit, vegetables, wholegrain and fibres in populations at risk of colorectal carcinoma, as well as the supplementation of specific nutrient blends.

Third chemopreventive strategy

The third new line of intervention focuses on the role of the ERs[27]. Since the discovery of ERs in the colonic tumour cells[28,29], several epidemiological and clinical studies have supported the idea that estrogens play a protective role in the pathogenesis of colorectal neoplastic lesions, suggesting their potential use in the prevention of colorectal cancer[16,30-33]. There is evidence of estrogens proliferative modulation not only on the usual estrogens responsive tissues[34] but also on other apparatuses[35].

Estrogens bind two types of receptors: estrogen receptor-alpha (ER-α), prevalent in the breast, bone, cardiovascular tissue, urogenital tract and central nervous system, and estrogen receptor-beta (ER-β), prevalent in the gut[36,37].

ER-β expression is significantly lower in colonic adenocarcinoma cells than in normal colonic epithelial cells and this reduction is directly correlated with the degree of tumour dedifferentiation[15]. Although ER expression has been widely investigated in colorectal cancer cells (CRCs)[15,38,39], few data are available about colorectal pre-

cancerous lesions.

Recently data confirm the involvement of ERs-β in colorectal carcinogenesis and suggest a possible explanation for the protective effect of oestrogens on cancer development[17-19]. They also further support the role of vegetable-rich diets in the prevention of bowel cancer, thanks to their high content of phytoestrogens[5].

In particular milk thistle, traditionally used as an antioxidant and antifibrotic agent in chronic liver disease[14], is the source of silymarin, an ER-β selective-agonist[15]. Silymarin has been documented to be an effective chemopreventive in the intestinal tumour progression[15,16,39]. The lignans, non-soluble dietary fibres has been reported to be similarly effective in the chemoprevention of CRC[17,40] most likely for their ability to absorb potential carcinogens in the intestinal lumen[17-18]. Barone *et al*[41] demonstrated with animal studies that ER-β expression is amenable to dietary modulation to regress and/or oppose the progression of the adenoma-adenocarcinoma sequence. In a randomized, double-blind and placebo controlled study in patients undergoing surveillance colonoscopy because of recurrent sporadic adenopolyposis, a two months supplementation of Eviendep® (5 g *bid*) was able to specifically induce the expression of the ERβ in the colon mucosa, with optimal tolerability and safety[42]. The same group recently demonstrated that the oral supplementation of Eviendep to patients undergoing surveillance colonoscopy because of recurrent sporadic adenopolyposis was able to significantly increase the expression of ER-β in the colon mucosa, with optimal tolerability and safety[42].

At the same time, Yamada *et al*[43] performed a study with capsule endoscopy and found that patients with duodenal polyps had a larger number of polyps in the small intestine than those without duodenal polyps. In our experiences 8 of the 11 patients enrolled were previously investigated by capsule endoscopy. In our subset of patients there was no evidence of polyps in the small intestine.

In conclusion, our study demonstrated for the first time that short-term (90 d) supplementation with Eviendep® in FAP patients with IPAA and with recurrent adenomas in the duodenal mucosa, resulted effective in reducing polyps number by 32% and size by 51%. Eviendep® was easy to manage and its daily use was well tolerated by the patients. Its safety was guaranteed by its composition. Each ingredient is blended into the composition in a lower dose than the one otherwise needed for the single component to similarly exert the desired effect, thus leading to synergistic and/or potentiating effect, with the added advantage of higher safety, even over long-term exposure. Further molecular studies are needed to better confirm the role of estrogens in the duodenal mucosa, but we do believe that they also play a central role in duodenal carcinogenesis in the colon.

COMMENTS

Background

Familial adenomatous polyposis (FAP) is a disease with autosomal dominant inheritance. Early prophylactic colorectal surgery changed the prognosis of patients with FAP, and duodenal cancer is nowadays the most important cause of death in FAP patients.

Research frontiers

The recent discover of the involvement of estrogen receptors (ERs)-β in colorectal carcinogenesis suggests a possible explanation for the protective effect of estrogens in cancer development. This also provided further support of the role of vegetable-rich diets in the prevention of bowel cancer, thanks to their high content of phytoestrogens (ER-β selective agonists).

Innovations and breakthroughs

Several epidemiological and clinical studies have supported the idea that estrogens play a protective role in the pathogenesis of colorectal neoplastic lesions, suggesting their potential use in the prevention of colorectal cancer. The aim of this study was to evaluate if dietary supplementation with phytoestrogens, selective agonists of the estrogen receptor, was able to prevent as well the progression of carcinogenesis in duodenal polyps.

Applications

Their study demonstrated that short-term supplementation with phytoestrogens in FAP patients with ileal pouch-anal anastomosis (IPAA) is effective in reducing duodenal polyps number and size.

Peer review

The authors investigated the effects of short-term (90 d) supplementation with Eviendep on the reduction of the number and size of duodenal polyps in FAP patients who had undergone IPAA.

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BRIEF ARTICLE

Fatty acids of erythrocyte membrane in acute pancreatitis patients

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Abstract

AIM: To evaluate changes in the fatty acid composition of erythrocyte membrane phospholipids during severe and mild acute pancreatitis (AP) of alcoholic and nonalcoholic etiology.

METHODS: All consecutive patients with a diagnosis of AP and onset of the disease within the last 72 h admitted to the Hospital of Lithuanian University of Health Sciences between June and December 2007 were included. According to the Acute Physiology and Chronic Health Evaluation (APACHE Ⅱ) scale, the patients were subdivided into the mild (APACHE Ⅱ score < 7, n = 22) and severe (APACHE Ⅱ score ⩾ 7, n = 17) AP groups. Healthy individuals (n = 26) were enrolled as controls. Blood samples were collected from patients on admission to the hospital. Fatty acids (FAs) were extracted from erythrocyte phospholipids and expressed as percentages of the total FAs present in the chromatogram. The concentrations of superoxide dismutase and glutathione peroxidase were measured in erythrocytes.

RESULTS: We found an increase in the percentages of saturated and monounsaturated FAs, a decrease in the percentages of total polyunsaturated FAs (PUFAs) and n-3 PUFAs in erythrocyte membrane phospholipids of AP patients compared with healthy controls. Palmitic (C16:0), palmitoleic (C16:1n7cis), arachidonic (C20:4n6), docosahexaenoic (DHA, C22:6n3), and docosapentaenoic (DPA, C22:5n3) acids were the major contributing factors. A decrease in the peroxidation and unsaturation indexes in AP patients as well as the severe and mild AP groups as compared with controls was observed. The concentrations of antioxidant enzymes in the mild AP group were lower than in the control group. In severe AP of nonalcoholic etiology, the percentages of arachidic (C20:0) and arachidonic (C20:4n6) acids were decreased as compared with the control group. The patients with mild AP of nonalcoholic etiology had the increased percentages of total saturated FAs and gama linoleic acid (C18:3n6) and the decreased percentages of elaidic (C18:1n9t), eicosapentaenoic acid (EPA, C20:5n3), DPA (C22:5n3), DHA (C22:6n3) as well as total and n-3 PUFAs in erythrocyte membrane phospholipids.

CONCLUSION: The composition of FAs in erythrocyte membranes is altered during AP. These changes are likely to be associated with alcohol consumption, inflammatory processes, and oxidative stress.

Key words: Acute pancreatitis; Alcohol; Fatty acids; Oxidative stress; Systemic inflammatory response syndrome

Core tip: The manuscript by Kuliaviene *et al* elucidates the changes of fatty acids in erythrocyte membrane phospholipids during acute pancreatitis. Alcohol may influence the increased percentage of saturated and monounsaturated fatty acids of erythrocyte membrane. Fatty acids that are linked with inflammatory processes change differently during severe and mild nonalcoholic acute pancreatitis. The decrease of pro-inflammatory acids is seen in severe acute pancreatitis while anti-inflammatory players decrease during mild acute pancreatitis. The antioxidant enzymes of erythrocytes change in mild but not severe pancreatitis group. Thus the erythrocyte membranes can reflect the inflammatory and oxidative processes of acute pancreatitis.

Kuliaviene I, Gulbinas A, Cremers J, Pundzius J, Kupcinskas L, Dambrauskas Z, Jansen E. Fatty acids of erythrocyte membrane in acute pancreatitis patients. *World J Gastroenterol* 2013; 19(34): 5678-5684 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i34/5678.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i34.5678

INTRODUCTION

Acute pancreatitis (AP) is a sudden inflammation of pancreas. About 20%-30% of patients develop severe forms of the disease manifesting with local and systemic complications. Acute pancreatitis carries an overall mortality rate of 10%-15%[1,2]. The main causes of death are associated with multiple organ failure and pancreatic infection[3,4]. The initial process of inflammation starts in the pancreas, but no strict correlation between pancreatic necrosis and organ failure has been reported[5]. Systemic inflammatory response is responsible for multiple organ failure and has the most considerable impact on the severity of acute pancreatitis and mortality from this disease[6].

The role of fatty acids (FAs) in the pathogenesis of AP is important but far from being clear. An increase the total serum free FA level is observed during AP[7]. Unsaturated FAs, especially polyunsaturated FAs (PUFAs), are liberated from pancreatic necrotic tissues and are responsible for the disturbance of FA profile in the serum of patients with AP[7,8]. The increased amount of unsaturated FAs in the necrotic pancreatic tissue and serum during AP is associated with multisystem organ failure and worse outcomes of patients[9]. Moreover, alcohol, an important etiological factor for pancreatitis, has an impact on the FA composition of serum and erythrocyte membranes[10-13]. Surprisingly, during alcohol-induced pancreatitis, the percentage of PUFAs is decreased in the serum FA profile in mild and moderate AP as well as chronic pancreatitis[14,15]. These data suggest that alcohol could play a specific role in the pathogenesis of pancreatitis.

FAs of cell membranes are precursors for lipid mediators and play an important role in the process of inflammation and oxidant status[16]. Experimental findings show that n-3 PUFAs may be beneficial in the prevention of oxidative stress-induced inflammation in pancreatitis[17]. Moreover, it influences the histological severity of AP[18-21]. Human studies also indicate likely clinical benefits of enteral feeding rich in n-3 PUFAs in patients with AP[22].

The aim of our study was to evaluate changes in the FA profile of erythrocyte membrane phospholipids and antioxidant enzymes of erythrocytes in patients with severe and mild AP, also of nonalcoholic etiology separately, in comparison with healthy individuals. We believe that erythrocyte membrane phospholipids can better reflect systemic changes caused by oxidative stress and inflammatory response in patients with AP comparing with FAs in serum, which are greatly influenced by necrotic changes in the pancreas and peripancreatic tissues. To our knowledge, no studies examining the FA composition of erythrocyte membrane phospholipids during AP have been carried out.

MATERIALS AND METHODS

Patients

All consecutive patients with a diagnosis of AP and onset of the disease within the last 72 h admitted to the Departments of Surgery and Gastroenterology at the Hospital of Lithuanian University of Health Sciences between June and December 2007 were included in this study. The diagnosis was established based on acute abdominal pain, at least 3-fold elevated levels of serum amylase, and typical radiological findings. According to the Acute Physiology and Chronic Health Evaluation (APACHE Ⅱ) scale, the patients were subdivided into the mild (APACHE Ⅱ score < 7, $n = 22$) and severe (APACHE Ⅱ score $\geqslant 7$, $n = 17$) AP groups. Healthy subjects ($n = 26$) without a past history of pancreatic diseases were enrolled as controls.

Fatty acid and antioxidant analysis

Peripheral blood samples were drawn from patients on admission to the hospital. Plasma and leukocytes were removed after centrifugation. Erythrocytes were washed and centrifuged twice. The samples were stored at -80 ℃ until analysis. The blood samples of the control group were subjected to the same procedure.

FA analysis was performed in the Laboratory for Health Protection Research, National Institute for Public Health and the Environment (The Netherlands), as described previously[23]. Briefly, 200 μL of erythrocytes was taken, and phospholipids were washed with distilled water and extracted with chloroform/methanol (1:1). The chloroform layer was evaporated, and the phospholipids were hydrolyzed and methylated simultaneously

Table 1 Demographic and clinical data of patients and controls

	AP	Severe AP	Mild AP	Control
Age, mean ± SD, yr	48.1 ± 15.5	50.2 ± 13.7	46.7 ± 16.8	42.07 ± 16.6
Men	68%	64%	70%	33%
AP etiology				
Alcoholic	35%	36%	35%	NA
Nonalcoholic	65%	64%	65%	NA
Death	6%	7%	5%	NA

Values are percentage unless otherwise stated. NA: Not applicable; AP: Acute pancreatitis.

with BF_3/MeOH for 60 min at 100 ℃. After extraction with hexane, the methylated FAs (FAME) were separated on a fused silica capillary column using a GC-3900 gas chromatograph with FID detection (Varian Assoc). The baseline separation of more than 50 FAME peaks was accomplished using FAME standards (Sigma) within 57 min. Individual FAs were expressed as percentages of the total FAs present in the chromatogram.

The concentrations of superoxide dismutase (SOD) and glutathione peroxidase (GPx) were measured in erythrocytes on an auto analyzer (LX-20 Pro, Beckman-Coulter, Woerden, Netherlands) with kits from Randox (Ransod and Ransel, Crumlin, United Kingdom).

Peroxidation and unsaturation index

The indexes were calculated according to the formulas used by Viviani *et al*[24]. The peroxidation index (PI) was determined from the percentages of monoenoic, dienoic, trienoic, tetraenoic, pentaenoic and hexanoic FAs according to the following formula: PI = [(%monoenoic × 0.025) + (%dienoic × 1) + (%trienoic × 2) + (%tetraenoic × 4) + (%pentaenoic × 6) + (%hexanoic × 8)].

The unsaturation index (UI) is also known as the index of hydrogen deficiency. It was calculated from the number of unsaturated double bonds of each FA: UI = [(%monoenoic × 1) + (%dienoic × 2) + (%trienoic × 3) + (%tetraenoic × 4) + (%pentaenoic × 5) + (%hexanoic × 6)].

Ethics

The study was approved by Kaunas Regional Ethics Committee for Biomedical Research (BE-2-47). All patients and healthy subjects provided written informed consent.

Statistical analysis

Statistical analysis was performed using SPSS® for Windows release 14.0 (SPSS, Chicago, IL, United States). The data are presented as mean ± SD. The Mann-Whitney test and one-way and two-way ANOVA tests were applied for analysis of variables. All statistical tests were two sided, and $P < 0.05$ was considered statistically significant.

RESULTS

The demographic characteristics of patients and controls are presented in Table 1. There was no difference in the FA composition of membrane phospholipids between men and women in the control group (data not shown).

Table 2 Percentages of saturated, monounsaturated and polyunsaturated fatty acids in erythrocyte membrane phospholipids

	AP	Alcoholic AP	Nonalcoholic AP	Control
Saturated fatty acid				
C14:0	0.26 ± 0.06	0.26 ± 0.06	0.26 ± 0.07	0.27 ± 0.06
C15:0	0.29 ± 0.05	0.27 ± 0.04	0.30 ± 0.06	0.30 ± 0.05
C16:0	23.43 ± 1.12[b]	23.80 ± 1.20[b]	23.22 ± 1.06[b]	22.17 ± 0.85
C17:0	0.27 ± 0.06[b]	0.23 ± 0.03[b]	0.29 ± 0.06	0.31 ± 0.03
C18:0	13.49 ± 0.70[a]	13.40 ± 0.54	13.54 ± 0.78	13.95 ± 0.70
C20:0	0.33 ± 0.10[b]	0.28 ± 0.06[b]	0.36 ± 0.09	0.40 ± 0.06
C21:0	0.04 ± 0.04	0.04 ± 0.06	0.04 ± 0.04	0.03 ± 0.03
C22:0	1.39 ± 0.33	1.15 ± 0.23[b]	1.47 ± 0.34	1.44 ± 0.26
C23:0	0.21 ± 0.06[a]	0.17 ± 0.04[b]	0.23 ± 0.06	0.24 ± 0.04
C24:0	4.44 ± 0.80	4.30 ± 0.77	4.41 ± 0.97	4.10 ± 0.73
Total	44.05 ± 1.47[a]	43.90 ± 1.00	44.13 ± 1.70	43.34 ± 0.90
Monounsaturated fatty acid				
C16:1n7*trans*	0.13 ± 0.02	0.12 ± 0.02	0.13 ± 0.02	0.14 ± 0.02
C16:1n9c	0.09 ± 0.06[b]	0.09 ± 0.03	0.09 ± 0.07	0.06 ± 0.03
C16:1n7c	0.56 ± 0.19[b]	0.68 ± 0.17[b]	0.49 ± 0.17[b]	0.33 ± 0.06
C18:1n9*trans*	0.11 ± 0.05[b]	0.10 ± 0.02[b]	0.12 ± 0.06[a]	0.16 ± 0.04
C18:1n7*trans*	0.28 ± 0.09[a]	0.23 ± 0.07[b]	0.30 ± 0.09	0.33 ± 0.09
C18:1n9c	13.02 ± 1.49	13.53 ± 1.05[a]	12.85 ± 1.88	12.42 ± 1.12
C18:1n7c	1.08 ± 0.17	1.09 ± 0.15	1.07 ± 0.20	1.03 ± 0.13
C22:1n9c	0.22 ± 0.05[a]	0.23 ± 0.06[a]	0.22 ± 0.05[a]	0.18 ± 0.05
C24:1	4.98 ± 0.69	4.86 ± 0.63	4.87 ± 0.86	4.58 ± 0.67
Total	20.49 ± 1.98[a]	20.9 3± 1.13[a]	20.16 ± 2.29	19.31 ± 1.02
PUFA				
Omega 3 PUFA	7.84 ± 1.71[b]	7.92 ± 1.74[a]	7.80 ± 1.74[b]	9.45 ± 1.30
C18:3n3	0.16 ± 0.05	0.18 ± 0.06	0.14 ± 0.04	0.15 ± 0.03
C20:5n3	0.91 ± 0.46	0.94 ± 0.46	0.82 ± 0.42	1.02 ± 0.4
C22:5n3	2.21 ± 0.33[a]	2.29 ± 0.36	2.17 ± 0.32[a]	2.39 ± 0.29
C22:6n3	4.61 ± 1.12[b]	4.50 ± 1.12[b]	4.67 ± 1.14[b]	5.89 ± 0.81
Omega 6 PUFA	26.99 ± 1.99	26.89 ± 1.40	27.51 ± 2.17	27.93 ± 1.58
C18:2n6c	10.16 ± 1.24	10.07 ± 1.01	10.43 ± 1.43	10.18 ± 1.27
C18:3n6	0.05 ± 0.02[b]	0.05 ± 0.02[b]	0.04 ± 0.02[a]	0.03 ± 0.01
C20:3n6	1.44 ± 0.26[a]	1.49 ± 0.27	1.41 ± 0.25	1.32 ± 0.34
C20:4n6	12.94 ± 1.06[a]	12.82 ± 0.82[a]	13.01 ± 1.18[a]	13.64 ± 0.99
C22:4n6	2.48 ± 0.58	2.46 ± 0.35	2.62 ± 0.60	2.63 ± 0.50
C16:3n4	0.05 ± 0.02[a]	0.04 ± 0.02[b]	0.06 ± 0.02	0.06 ± 0.02
C20:2	0.28 ± 0.06[a]	0.27 ± 0.05	0.29 ± 0.06[a]	0.25 ± 0.03
C22:2	0.05 ± 0.02	0.05 ± 0.01	0.06 ± 0.02	0.05 ± 0.02
Total PUFA	35.51 ± 1.95[b]	35.17 ± 1.37[b]	35.71 ± 2.21[b]	37.34 ± 1.30

Results are presented as mean ± SD. Percentages of saturated, monounsaturated and polyunsaturated fatty acids (PUFA) in erythrocyte membrane phospholipids in patients with acute pancreatitis (AP) of alcoholic and nonalcoholic etiology. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control group.

As shown in Table 2, the percentage of saturated FAs in erythrocyte membrane phospholipids was greater in the patients with AP than the control group. Palmitic acid (C16:0) had a major impact on the increase. The percentages of monounsaturated FAs were also increased, and *cis*-isomers, especially palmitoleic (C16:1n7*cis*) and erucic (C22:1n9*cis*) acids, mostly contributed to the change. We found a decreased percentage of some *trans* monounsaturated FAs in erythrocyte membrane phospholipids. Those were elaidic (C8:1n9*trans*) and vaccenic (C18:1n7 *trans*) acids (Table 2).

The percentages of total and n-3 PUFAs were decreased in erythrocyte membrane phospholipids of AP

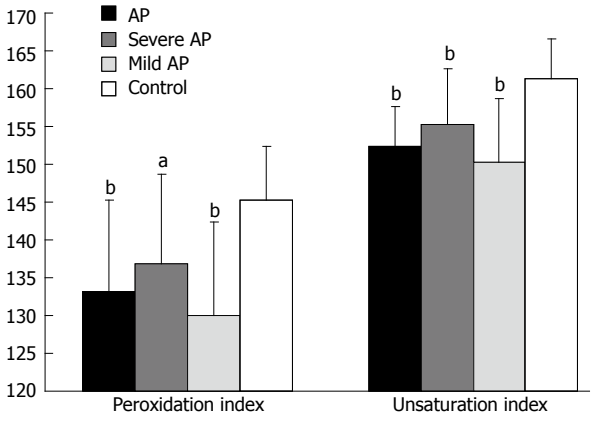

Figure 1 Peroxidation and unsaturation indexes of fatty acids of erythrocyte membrane phospholipids in the acute pancreatitis, severe acute pancreatitis, mild acute pancreatitis, and control groups. A significant decrease of peroxidation index and unsaturation index in acute pancreatitis (AP) patients compared with controls was observed. Bars represent mean values with standard deviation. The extent of change in the indexes in mild AP was greater than in severe AP patients comparing with controls ($^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group).

patients. This was particularly caused by the decreased percentages of docosahexaenoic (DHA C22:6n3) and docosapentaenoic (DPA C22:5n3) acids. The percentage of arachidonic acid (AA, C20:4n6) was decreased in the patients with AP comparing with the controls, though the percentages of gama-linoleic (C18:3n6) and dihomo gama linoleic (C20:3n6) acids were increased (Table 2).

A decrease of PI and UI in AP patients as well as the severe and mild AP groups compared with the controls was observed. The extent of change in the indexes in the mild AP group was greater than in the severe AP group compared with controls (Figure 1). The concentrations of SOD and GPx in the mild AP group were lower comparing with the control group (321.55 ± 75.19 μmol/mL and 10059.21 ± 2666.96 U/L *vs* 384.88 ± 42.21 μmol/mL and 11649.09 ± 1844.75 U/L, $P < 0.001$ and $P < 0.05$, respectively). To rule out the impact of alcohol in the changes of FAs in erythrocyte membrane phospholipids of AP patients, we analyzed AP of non-alcoholic etiology (Table 3). In the severe AP group, the percentages of arachidic acid (C20:0) and AA (C20:4n6) were decreased as compared with the control group. In the mild AP group, an increase in the percentages of total saturated FAs and gama linoleic acid (C18:3n6) and a decrease in the percentages of elaidic (C18:1n9t), EPA (C20:5n3), DPA (C22:5n3), DHA (C22:6n3), total PUFAs, and omega 3 PUFAs in erythrocyte membrane phospholipids were recorded. The change in the concentrations of antioxidant enzymes showed the same pattern: they were decreased in patients with mild AP and showed no change in the severe AP group. The changes in the PI and the UI appeared to be greater in the mild AP than severe AP group as compared with the control group.

DISCUSSION

This study has analyzed the impact of systemic inflammatory response and oxidative stress on the FA composition of erythrocyte membranes and the concentrations of enzymes in patients with AP. The initial generation of ROS and inflammatory events occur in the pancreas, but systemic changes have a crucial impact on the severity and fatal outcomes of AP[6]. A better understanding of these systemic processes occurring during AP could help identifying new therapeutic treatment options and escaping undesirable complications or fatal outcomes.

In our study, we found that the FA composition of erythrocyte membrane phospholipids was significantly altered during AP compared with controls mainly because of the increased percentages of saturated and monounsaturated acids, namely palmitic and palmitoleic, and a decreased percentage of PUFAs. Contrary, Sztefko *et al*[7] found that the proportion of saturated and monounsaturated acids was decreased and the proportion of PUFAs was increased in the serum levels of free FAs in patients with AP. An increase in the percentage of PUFAs in the necrotic pancreatic tissue has also been reported[8]. On the other hand, in severe sepsis, a similar pathology with systemic inflammatory response syndrome, the lower proportions of PUFAs and the greater proportions of monounsaturated FAs in erythrocyte phospholipids have been documented[25]. These findings suggest that the FA composition of erythrocyte membrane phospholipids may reflect not only the direct events in the pancreas, but also the systemic response syndrome during AP.

Alcohol consumption can be associated with the higher percentages of saturated and monounsaturated FAs, such as palmitic and oleic acids, and the lower percentages of PUFAs, especially DHAs and arachidonic acid, in serum and membranes[10-13]. Alcoholics have also been show to have a disturbed oxidant status of plasma and erythrocyte enzymes[26-28]. In the study by Khan *et al*[14], the authors showed the increased percentages of saturated palmitic and monounsaturated FAs as well as the decreased percentages of some PUFAs in serum of patients with alcohol-induced AP comparing with alcoholic controls. Moreover, Gabianelli *et al*[29] reported that ethanol can have a direct toxic effect on erythrocyte membranes and antioxidant systems of the cells. These findings indicate that alcohol may have an impact on the FA composition of erythrocyte membrane phospholipids. Thus, the increased percentages of saturated and monounsaturated FAs in our study could partly be explained by etiological factors, most probably alcohol.

To rule out the impact of alcohol and to study the influence of inflammatory and oxidative processes during AP on the phospholipid composition of erythrocyte membranes, we analyzed patients with AP of nonalcoholic etiology. The PUFAs of cell membranes are precursors for prostaglandins and other lipid mediators of inflammatory process[16]. Arachidonic acid is the main proinflammatory actor. Meanwhile, EPA, DHA, and possibly DPA are precursors for products with anti-inflammatory and proresolving functions[30,31]. We found that in the severe AP group, the percentage of proinflammatory arachidonic acid was significantly decreased, and

Table 3 Percentages of fatty acids

	Nonalcoholic		Control
	Severe AP	Mild AP	
C20:0	0.32 ± 0.09[d]	0.39 ± 0.09	0.40 ± 0.06
C20:4n6	12.89 ± 0.83[c]	13.09 ± 1.40	13.64 ± 0.99
SFA	43.53 ± 1.29	44.54 ± 1.86[c]	43.34 ± 0.90
C18:3n6	0.04 ± 0.02	0.05 ± 0.02[c]	0.03 ± 0.01
C18:1n9t	0.13 ± 0.04	0.12 ± 0.07[d]	0.16 ± 0.04
C20:5n3	1.03 ± 0.56	0.67 ± 0.18[d]	1.02 ± 0.4
C22:5n3	2.28 ± 0.22	2.10 ± 0.36[c]	2.39 ± 0.29
C22:6n3	5.29 ± 1.02	4.24 ± 1.05[d]	5.89 ± 0.81
n-3 PUFA	8.76 ± 1.73[a]	7.14 ± 1.45[d]	9.45 ± 1.30
Total PUFA	36.87 ± 1.62[a]	34.91 ± 2.26[d]	37.34 ± 1.30
SOD	385.37 ± 21.37[a]	314.90 ± 86.04[d]	384.88 ± 42.21
GPx	12557.07 ± 2836.06[a]	9370.85 ± 2196.13[d]	11649.09 ± 1844.75
PI	138.49 ± 10.34[a,c]	128.14 ± 10.74[d]	146.19 ± 7.08
UI	156.67 ± 7.28[a,c]	148.99 ± 8.04[d]	161.70 ± 4.90

Results are presented as mean ± SD. Percentages of fatty acids in erythrocyte membrane phospholipids, peroxidation and unsaturation indexes, and concentrations of superoxide dismutase (SOD, μmol/mL) and glutathione peroxidase (GPx, U/L) in patients with acute pancreatitis (AP) of nonalcoholic etiology. [a]$P < 0.05$ comparing mild and severe AP; [c]$P < 0.05$, [d]$P < 0.01$ *vs* control group. PUFA: Polyunsaturated fatty acids; SFA: Saturated fatty acids.

in the mild AP group, a decrease in the percentages of anti-inflammatory players (EPA, DHA, and DPA) was seen as compared with controls. It is now thought that saturated FAs could also be involved in the inflammatory process[32,33]. We also found a significant increase in the percentage of total saturated FAs in the mild but not severe AP group. Erythrocytes are not usually considered to be active players in the inflammatory process, but our study showed that the changes in the percentage of FAs in erythrocyte membrane phospholipids were different during mild and severe AP, therefore, we hypothesize that the composition of erythrocyte membrane phospholipids may reflect the inflammatory processes and the severity of the disease.

Oxidative stress plays a central role in the development of pancreatic inflammation and extra pancreatic complications[34-36]. The changes of the FA composition of erythrocyte membrane phospholipids could be affected from "the outside" as PUFAs of erythrocyte membrane phospholipids are extremely sensitive to oxidation[37]. SOD and GPx are important components of enzymatic antioxidant defense[37]. We found that the levels of antioxidant enzymes in erythrocytes were also altered significantly in the mild but not severe AP group comparing with controls. There were significant differences in the PI and the UI mainly because of the different percentage of PUFAs in erythrocyte membrane phospholipids in the severe and mild AP groups comparing with controls. Moreover, there was a significant difference between the severe and mild AP groups. This suggests that oxidative stress might be involved in the changes of the FA composition of erythrocyte membrane phospholipids and systemic inflammatory response.

It was unexpected to find the changes of PI and UI to be more apparent in the mild AP than severe AP group comparing with controls. It is known that systemic response followed by organ failure influences the severity and outcome of AP more than the events in the pancreas itself[5]. The similar phenomenon was also noticed in cytokine expression during mild and severe AP[38]. We hypothesize that these findings could reflect the disproportion of pro- and anti-inflammatory processes during severe AP possibly associated with oxidative stress. The mechanisms of these processes are still not clear and remain to be elucidated.

The composition of FAs in erythrocyte membranes is altered during AP. These changes are likely to be associated with alcohol intake as an etiological factor for AP, and systemic inflammatory processes and oxidative stress after the onset of the disease could influence the changes.

COMMENTS

Background

Acute pancreatitis carries an overall mortality rate of 10%-15%. Systemic inflammatory response has the most considerable impact on the severity of acute pancreatitis and mortality from this disease. Fatty acids of cell membranes are precursors for lipid mediators and play an important role in the process of inflammation and oxidant status. Erythrocyte membrane phospholipids can reflect systemic changes caused by oxidative stress and inflammatory response in patients with acute pancreatitis.

Research frontiers

The role of fatty acids in the pathogenesis of acute pancreatitis is important but far from being clear. Fatty acids are the components of membrane phospholipids. They are responsible for inflammatory and oxidative processes. Free fatty acids in serum are associated with necrotic lesions in the pancreas and peripancreatic tissues. Moreover, alcohol, an important etiological factor for pancreatitis, has an impact on the fatty acid composition of serum and erythrocyte membranes.

Innovations and breakthroughs

The earlier studies of alterations in the fatty acid composition during acute pancreatitis mainly investigated the fatty acid composition in serum that is greatly influenced by necrotic changes in the pancreas and peripancreatic tissues. Authors believe that erythrocyte membrane phospholipids can better reflect systemic changes caused by oxidative stress and inflammatory response as well as alcohol impact in patients with acute pancreatitis. To the knowledge, no studies examining the fatty acid composition of membranes during acute pan-

creatitis have bee carried out.

Applications

This study elucidates the pathogenesis of acute pancreatitis, especially the systemic and oxidative processes that are of high importance in the severity of acute pancreatitis and mortality from this disease.

Terminology

Fatty acids are the components of phospholipids that form the lipid bilayers of cell membranes. Fatty acids of membranes are precursors for lipid mediators of inflammatory response syndrome. Oxidative stress is a disturbance of the pro-oxidant-antioxidant balance in favor of the former, leading to potential damage, and is associated with many chronic and acute inflammatory conditions.

Peer review

This study evaluates changes in fatty acids of erythrocyte membrane phospholipids during mild and severe acute pancreatitis, of alcohol and non-alcohol etiology. The study is a prospective one and Acute Physiology and Chronic Health Evaluation Ⅱ score was used to classify patients into mild (n = 22 patients) and severe (n = 17 patients). Some 26 healthy individuals were enrolled as control. This is a well conducted prospective study, and to my knowledge this is the first study that examines the fatty acid profile of the erythrocyte membrane in acute pancreatitis.

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BRIEF ARTICLE

Diversity of *Helicobacter pylori* genotypes in Iranian patients with different gastroduodenal disorders

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Abstract

AIM: To investigate the diversity of *Helicobacter pylori* (*H. pylori*) genotypes and correlations with disease outcomes in an Iranian population with different gastroduodenal disorders.

METHODS: Isolates of *H. pylori* from patients with different gastroduodenal disorders were analyzed after culture and identification by phenotypic and genotypic methods. Genomic DNA was extracted with the QIAamp DNA mini kit (Qiagen, Germany). After DNA extraction, genotyping was done for *cagA*, *vacA* (s and m regions), *iceA* (*iceA1*, *iceA2*) and *babA* with specific primers for each allele using polymerase chain reaction (PCR). All patients' pathologic and clinical data and their relation with known genotypes were analyzed by using SPSS version 19.0 software. χ^2 test and Fisher's exact test were used to assess relationships between categorical variables. The level of statistical significance was set at $P < 0.05$.

RESULTS: A total of 71 isolates from 177 patients with different gastroduodenal disorders were obtained. Based on analysis of the *cagA* gene (positive or negative), *vacA* s-region (s_1 or s_2), *vacA* m-region (m_1 or m_2), *iceA* allelic type (*iceA1* and *iceA2*) and *babA* gene (positive or negative), twenty different genotypic combinations were recognized. The prevalence of *cagA*, *vacA s1*, *vacA s2*, *vacA m1*, *vacA m2*, *iceA1*, *iceA2*, *iceA1*+*iceA2* and *babA* were 62%, 78.9%, 19.7%, 21.1%, 78.9%, 15.5%, 22.5%, 40.8% and 95.8%, respectively. Interestingly, evaluation of PCR results for *cagA* in 6 patients showed simultaneous existence of *cagA* variants according to their size diversities that proposed mixed infection in these patients. The most prevalent genotype in *cagA*-positive isolates was *cagA*$^+$/*vacAs1m2*/*iceA1*+*A2*/*babA*+ and in *cagA*-negative isolates was *cagA*$^-$/*vacAs1m2*/*iceA*-/*babA*+. There were no relationships between the studied genes and histo-

pathological findings (*H. pylori* density, neutrophil activity, lymphoid aggregation in lamina propria and glandular atrophy). The strains which carry *cagA*, *vacAs1/m1*, *iceA2* and *babA* genes showed significant associations with severe active chronic gastritis (P = 0.011, 0.025, 0.020 and 0.031, respectively). The *vacAs1* genotype had significant correlation with the presence of the *cagA* gene (P = 0.013). Also, babA genotype showed associations with *cagA* (P = 0.024). In the combined genotypes, only *cagA*$^+$/*vacAs1m1*/*iceA2*/*babA*+ genotype showed correlation with severe active chronic gastritis (P = 0.025).

CONCLUSION: This genotyping panel can be a useful tool for detection of virulent *H. pylori* isolates and can provide valuable guidance for prediction of the clinical outcomes.

Key words: *Helicobacter pylori*; *cagA*; *vacA*; *iceA*; *babA*

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INTRODUCTION

Infection with *Helicobacter pylori (H. pylori)* causes different clinical disorders such as persistent gastritis, peptic ulcers and mucosa associated lymphoid tissue (MALT) lymphoma. Current studies suggest that *H. pylori* infection may be a crucial risk factor in the development of gastric cancer[1,2]. In this regard, this pathogen has been categorized as a group I carcinogen by the International Agency for Research on Cancer[3]. The detailed reasons for these different clinical outcomes are unknown, but they may be related to host genetic factors, exposure to environmental factors (*e.g.*, diet, drug usage, acidity of the stomach and smoking) and to the bacterial genotypes[4]. *H. pylori* shows extensive genetic diversity and this variability has a crucial role in pathogenesis of this bacterium[5]. Several *H. pylori* virulence factor genes related to the risk of gastroduodenal disorders, including *cagA*, *vacA*, *babA* and *iceA*, have been proposed[6]. A tremendous number of studies have proved that CagA and VacA producing strains are related to severe clinical outcomes[7]. In addition to *cagA* and *vacA*, the other *H. pylori* virulence factors, such as *iceA* and *babA,* also showed such associations in some studies[8,9]. Beyond the role of these factors in progression of the disease, there are several papers which reported a relationship between failure of *H. pylori* eradication therapy and the strains' virulence factor genotypes[10]. Analysis of genetic structure of virulence factors among the isolates from different geographic regions will provide new insights regarding the pathogenesis and treatment of *H. pylori* infection. *H. pylori* genotyping may have multiple roles including impact on the cure rates of eradication therapy[10], determination of clinical outcomes[11], tracking human migration[12,13] and recently, the prediction of progression of gastric preneoplastic lesions[14]. The distribution pattern of *H. pylori* genotypes and its correlation with disease outcome shows geographic differences. The aim of this study was to assess the diversity of *H. pylori* genotypes in an Iranian population to determine genotypically the *H. pylori* isolates more associated with different gastroduodenal disorders.

MATERIALS AND METHODS

Clinical specimens

Three gastric biopsies (two were used for histological examination and one for culture) were obtained from 177 adult patients undergoing routine diagnostic endoscopy referred to the Endoscopy Centre of Taleghani Hospital of Tehran, Iran, after obtaining informed consent. All subjects answered questionnaires related to age, sex, gastric or duodenal peptic ulcer diseases upon endoscopy.

Culture

Antral or body biopsy specimens from each patient were kept in transport medium consisting of thioglycolate with 1.3 g/L agar (Merck) and 3% yeast extract (Oxoid). The endoscopic biopsy specimens were cut into small pieces, homogenized with a sterile scalpel and were smeared on the surface of Brucella agar plates supplemented with 7% horse blood and Campylobacter selective supplement (vancomycin 2.0 mg, polymyxin 0.05 mg, trimethoprim 1.0 mg) and amphotericin B (2.5 mg/L). Incubation was performed in microaerophilic conditions at 37 ℃ for 5-7 d. Identification of *H. pylori* isolates was performed by analyzing colony morphology, Gram staining, oxidase, catalase and urease activities and *H. pylori*-specific polymerase chain reaction (PCR) (*glmM)*. The isolates were preserved in BHI broth containing 20% glycerol and 10% fetal calf serum and stored at -70 ℃.

DNA extraction

Genomic DNA was extracted with the QIAamp DNA mini kit (Qiagen, Germany) according to the manufacturer's instructions. The DNA was stored at -20 ℃ until used for molecular studies.

H. pylori genotyping

After DNA extraction, polymerase chain reactions (PCR) were performed in a volume of 25 μL containing 1 × PCR buffer, 1 μmol/L of each primer, 1 μL of genomic DNA (approximately 150 ng), 200 μmol/L of dNTPs mix, 2 mmol/L of $MgCl_2$, and 0.05 U/μL Taq DNA polymerase. PCR amplifications were performed in an automated thermal cycler (AG 22331; Eppendorf, Hamburg, Germany) under the following conditions: for *vacA s/m*: 33 cycles of 1 min at 94 ℃, 33 s at 55 ℃, and 1 min at 72 ℃; for *cagA*: 33 cycles of 1 min at 94 ℃, 1 min at

Table 1 Primers used in this study

Gene	Primers (5′→3′)	PCR product (bp)	Annealing temperature (℃)	Ref.
vacA (s_1/s_2)	VA1F: ATGGAAATACAACAAACACAc VA1R: CTGCTTGAATGCGCCAAAC	259-286	55	[6]
vacA (m_1/m_2)	VACm1m2F: CAATCTGTCCAATCAAGCGAG VACm1m2R: GCGTCAAAATAATTCCAAGG	567-642	55	[15]
cagA	CagAF: AATACACCAACGCCTCCAAG CagAR: TTGTTGCCGCTTTTGCTCTC	400	59	[16]
iceA$_1$	iceA1F: TATTTCTGGAACTTGCGCAACCTGAT M.Hpy1R: GGCCTACAACCGCATGGATAT	approximately 900	58	[17]
iceA$_2$	iceA2 F: CGGCTGTAGGCACTAAAGCTA iceA2 R: TCAATCCTATGTGAAACAATGATCGTT	approximately 800	58	[17]
babA	babAF: CCAAACGAAACAAAAAGCGT babAR: GCTTGTGTAAAAGCCGTCGT	271	58	[18]
glmM	GlmM2-F GGATAAGCTTTTAGGGGTGTTAGGGG GlmM1-R GCTTACTTTCTAACACTAACGCGC	296	52	[19]

59 ℃, and 1 min at 72 ℃; for *iceA*$_1$/*A*$_2$: 33 cycles of 1 min at 94 ℃, 40 s at 58 ℃, and 1 min at 72 ℃, and for *babA*: 35 cycles of 1 min at 94 ℃, 40 s at 58 ℃ and 1 min at 72 ℃. The amplified genes were detected by electrophoresis in a 1.2% agarose gel with ethidium bromide. Table 1 summarizes the primer sequences, annealing temperatures and the expected size of the PCR products.

Histopathological evaluation

Sections were stained with hematoxylin and eosin for analysis of *H. pylori*-related histology by an expert pathologist. Then the grade of gastritis was scored based on the updated Sydney System.

Statistical analysis

Data were analyzed by using SPSS version 19.0.0 software (IBM, IL, United States). χ^2 test and Fisher's exact test were used to assess relationships between categorical variables. The level of statistical significance was set at $P < 0.05$.

RESULTS

Infection rates and clinical disorders

A total of 71 isolates from 177 patients (parenthesis approximately 40%) with different gastroduodenal disorders were obtained. The *H. pylori*-positive patients consisted of 24 males and 47 females, with their ages ranging between 19 and 85 years (mean age, 66 years). All of the isolates showed positive results for the common identification test and *H. pylori*-specific PCR (*glmM*). Most of the infected patients suffered from chronic gastritis (84.6%), while the others showed duodenitis (9.8%), intestinal metaplasia (2.8%), hyperplasia (1.4%) and gastric cancer diseases (1.4%) (Table 2).

Allelic diversities in main putative virulence markers

***cagA* genotyping:** The 400-bp PCR product indicating the presence of the *cagA* gene was obtained in 44 isolates (62%) and 27 (38%) were negative. Interestingly, evaluation of PCR results for *cagA* in 6 patients showed simultaneous existence of *cagA* variants according to their size diversities.

***vacA* genotyping:** The frequency of *vacA s*$_1$, *vacA s*$_2$, *vacA m*$_1$ and *vacA m*$_2$ were 78.9%, 19.7%, 21.1% and 78.9%, respectively. Only one isolate was *vacA som*$_2$ (with no PCR product for s region).

***iceA* genotyping:** Sole existence of *iceA*$_1$ genotype was detected in 15.5% and *iceA*$_2$ genotype in 22.5% of the colonized patients. Interestingly, out of the total studied samples, 40.8% were infected with both *iceA*$_1$ and *iceA*$_2$ genotypes and 21.1% were negative for these genes.

***babA* genotyping:** *babA* was found in 68 of the patients (95.8%); however, three patients (4.2%) did not show this allelic variant (Figure 1).

Correlation of *H. pylori* genotypes with pathological data, patients' age and clinical outcome

Combination of genotypes: Based on the analysis of the *cagA* gene (positive or negative), *vacA* s-region (s_1 or s_2), *vacA* m-region (m_1 or m_2), *iceA* allelic types (*iceA*$_1$ and *iceA*$_2$) and *babA* (positive or negative), twenty different genotypic combinations were recognized. The most prevalent genotype in *cagA* positive isolates was *cagA*$^+$/*vacAs*$_1$*m*$_2$/*iceA*$_1$+*A*$_2$+/*babA*+ and in *cagA* negative isolates was *cagA*$^-$/*vacAs*$_1$*m*$_2$/*iceA*-/*babA*+ (Figure 2).

***Helicobacter pylori* density, neutrophil activity, lymphoid aggregation in lamina propria and glandular atrophy:** There was no significant relationship between *cagA* positivity and *H. pylori* density, neutrophil activity, lymphoid aggregation in lamina propria and glandular atrophy in the biopsies. Also no relationships were found between other genes and these histopathological findings.

Patients' age: There was no significant relationship between the genotypes, clinical and pathological data and patients' age.

Chronic gastritis: The gastritis was scored as severe ac-

Table 2 Association of combined genotypes with pathological conditions in *Helicobacter pylori* isolates

Combination of genotypes	SCG	SACG[2]	MACG	MiACG	MCG	H	M	GC	D	Total	*P* value[1]
cagA$^+$/*vacAs1m2*/*iceA1*+*iceA2*/*babA*+	1	12	2	0	0	0	1	0	1	17	
cagA$^+$/*vacAs1m1*/*iceA2*/*babA*+	0	3	0	0	0	0	0	0	1	4	0.025[2]
cagA$^+$/*vacAs1m2*/*iceA1*/*babA*+	0	3	1	0	0	0	1	0	2	7	
cagA$^+$/*vacAs1m1*/*iceA1*+*iceA2*/*babA*+	0	6	1	0	1	0	0	0	0	8	
cagA$^+$/*vacAs2m2*/*iceA1*+*iceA2*/*babA*+	0	1	1	0	0	0	0	0	0	2	
cagA$^+$/*vacAs0m2*/*iceA2*/*babA*+	0	0	0	0	0	0	0	0	1	1	
cagA$^+$/*vacAs1m2*/*iceA2*/*babA*+	0	1	0	0	0	0	0	1	0	2	
cagA$^+$/*vacAs2m2*/*iceA1*/*babA*+	0	0	1	0	0	0	0	0	0	1	
cagA$^+$/*vacAs2m2*/*iceA*-/*babA*+	0	1	0	0	0	0	0	0	0	1	
cagA$^+$/*vacAs1m2*/*iceA*-/*babA*+	0	1	0	0	0	0	0	0	0	1	
cagA$^-$/*vacAs1m2*/*iceA*-/*babA*+	0	3	2	0	1	0	0	0	1	7	
cagA$^-$/*vacAs1m1*/*iceA*-/*babA*+	0	1	0	0	1	0	0	0	0	2	
cagA$^-$/*vacAs2m2*/*iceA2*/*babA*+	1	0	0	0	2	0	0	0	0	3	
cagA$^-$/*vacAs1m2*/*iceA2*/*babA*+	0	2	2	0	0	1	0	0	0	5	
cagA$^-$/*vacAs2m2*/*iceA1*/*babA*+	0	1	0	0	0	0	0	0	0	1	
cagA$^-$/*vacAs1m1*/*iceA2*/*babA*+	0	0	1	0	0	0	0	0	0	1	
cagA$^-$/*vacAs2m2*/*iceA*-/*babA*+	0	3	1	0	0	0	0	0	0	4	
cagA$^-$/*vacAs1m2*/*iceA1*+*iceA2*/*babA*+	0	1	0	0	0	0	0	0	0	1	
cagA$^-$/*vacAs1m2*/*iceA1*+*iceA2*/*babA*-	0	0	0	1	0	0	0	0	0	1	
cagA$^-$/*vacAs2m2*/*iceA1*/*babA*-	0	0	1	0	0	0	0	0	1	2	
Total	2	39	13	1	5	1	2	1	7	71	

[1]Only $P < 0.05$ are indicated; [2]This P value is related to severe active chronic gastritis (SACG). SCG: Severe chronic gastritis; MACG: Moderate active chronic gastritis; MiACG: Mild active chronic gastritis; MCG: Moderate chronic gastritis; H: Hyperplasia; M: Metaplasia; GC: Gastric cancer; D: Duodenitis.

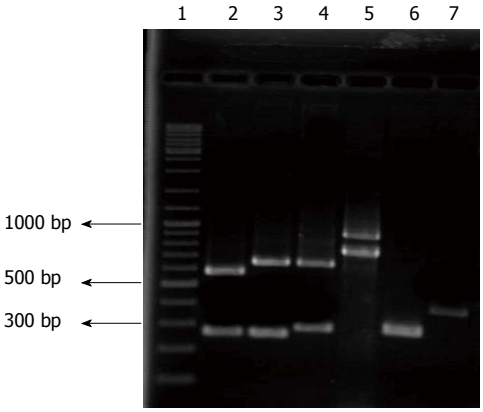

Figure 1 Polymerase chain reaction products of the main putative virulence markers. Lane 1: DNA ladder mix; Lane 2: *vacAs1m1* genotype; Lane 3: *vacAs1m2* genotype; Lane 4: *vacAs2m2* genotype; Lane 5: *iceA1*+*iceA2* genotype; Lane 6: *babA* genotype; Lane 7: *cagA* genotype

tive chronic gastritis, moderate active chronic gastritis, mild active chronic gastritis, severe chronic gastritis and moderate chronic gastritis. The strains which carried the *cagA* gene showed significant associations with severe active chronic gastritis ($P = 0.011$). Also, the strains which carried the *vacA* s_1/m_1 gene showed significant associations with severe active chronic gastritis ($P = 0.025$). *babA* ($P = 0.031$) and *iceA*$_2$ ($P = 0.020$) also had significant correlation with severe active chronic gastritis. In the combined genotypes this association was observed for *cagA*$^+$/*vacA*s_1m_1/*iceA*$_2$/*babA*+ genotype in the case of severe active chronic gastritis ($P = 0.025$).

Genotype correlation: Interestingly, the *vacA* s_1 genotype had significant correlation with the presence of the *cagA* gene ($P = 0.013$). Also *babA* genotype showed this association in *cagA* positive isolates ($P = 0.024$).

DISCUSSION

H. pylori infection is usually present in 60%-80% of gastric and 95% of duodenal ulcers. However, some conditions affect infection rate of this bacterium in different geographic and socioeconomic regions. The prevalence of infection is typically higher in developing countries (greater than 80%) and lower in the developed ones (typically less than 40%)[20]. It has been demonstrated that the prevalence of *H. pylori* infection in developing countries with low socioeconomic status and poor management of drinking water is much higher (> 80%) than that in developed countries (< 60%)[21]. In our study the recovery rate of *H. pylori* was 40% which shows the improvement in the living conditions and hygiene in Iran that has also been reported recently[22].

H. pylori can be divided into *cagA*-positive and *cagA*-negative strains, and there is increasing evidence that infection with *cagA*-positive isolates is associated with a greater risk of adverse clinical outcomes than infections with strains lacking this gene. In the current study, the strains which carried the *cagA* gene showed significant associations with severe active chronic gastritis. Interestingly, the prevalence of the *cagA*-positive strain differs among different countries, and more than 90% of *H. pylori* strains are *cagA*-positive in East Asian countries, irrespective of clinical presentation[23]. Sasaki *et al*[24] showed that among *H. pylori* DNA-positive samples, *cagA* was detected in 45.9% from Ecuador and 20.0% from Panama.

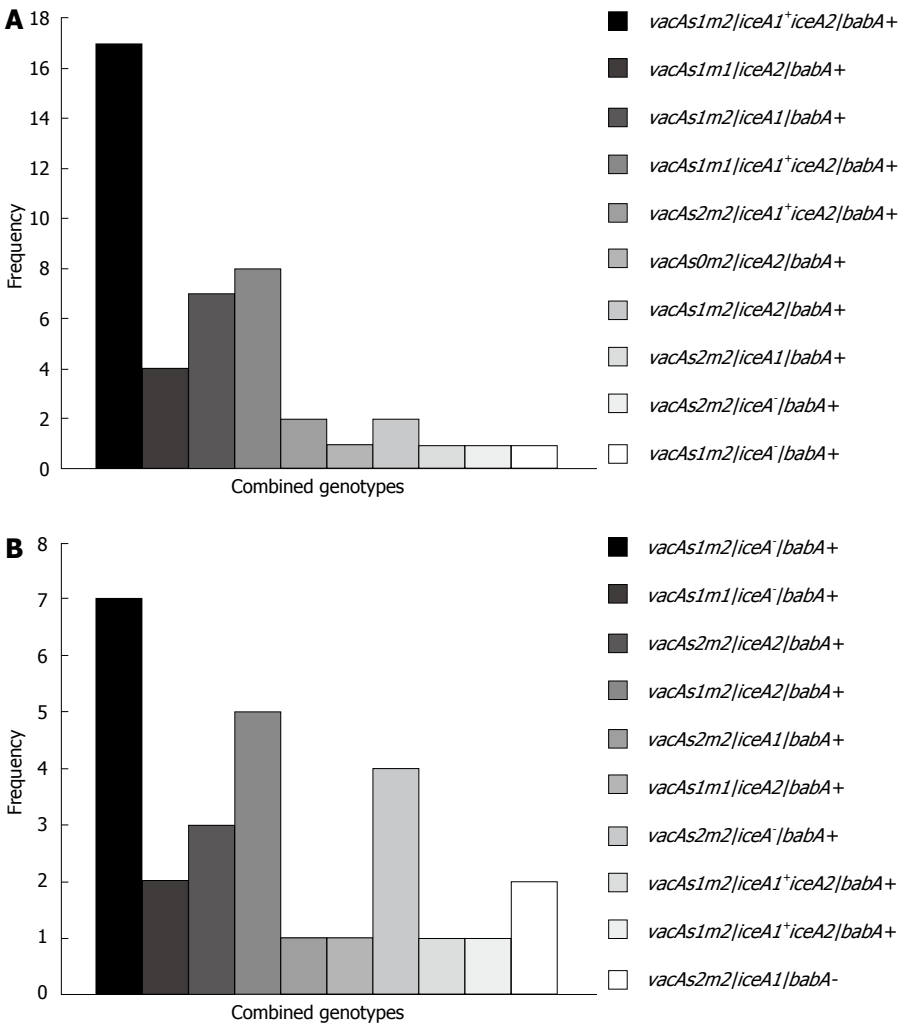

Figure 2 The frequency of combined genotypes. A: Combined *vacA*, *iceA* and *babA* genotypes in 44 *cagA* positive isolate; B: Combined *vacA*, *iceA* and *babA* genotypes in 27 *cagA* negative isolates.

In our study the prevalence of *cagA*-positive isolates is 62% which is less than other Asian countries and more than other countries (*e.g.*, Ecuador, Panama). According to Watada *et al*[25] study, the prevalence of *cagA* was 65.5% in Colombia and 100% in Japan, which showed that the prevalence of this gene in our study is similar to the Colombian isolates. In another study conducted in Bulgaria, the prevalence of *cagA* was 84.9% which is more than our results[26]. Interestingly, we had 6 isolates which had two different sizes of *cagA* simultaneously, showing the occurrence of mixed infection in these patients.

Variations of *vacA* are associated with different risks of gastrointestinal disorders. In general, *vacA* s_1 and m_1 genotypes produce a large amount of toxin, whereas s_2 and m_2 genotypes show little or no toxin production[27]. Recently, a third polymorphic determinant of vacuolating activity has been described as located between the s-region and m-region, an intermediate (i) region[28]. The frequency of the *vacA* s_1 and *vacA* m_1 genotypes in the Middle Eastern countries was found to be 71.5% and 32.8%, respectively[11], which is in concordance with our study. We did not detect any *vacA* s_2m_1 genotypes in our isolates which has been reported to be rare[23]. The *vacA* s_1 and m_1 genotypes have been reported to be associated with *H. pylori*-related diseases; however *vacA* s_2 and m_2 strains are rarely associated with peptic ulcer and gastric cancer because of their low or non-vacuolating activities[23]. Genotyping of *vacA* will be useful in screening individuals for risk factors associated with gastric cancer and peptic ulcer development. Asrat *et al*[29] showed that *vacAs1m1* genotype was the most common genotype in Ethiopian adult dyspeptic patients, and also *vacA*- and *cagA*-positive *H. pylori* strains were detected to a higher degree in patients with chronic active gastritis. Interestingly, similar to our results, correlation of the *vacA* s_1 genotype with the presence of the *cagA* gene was reported by Atherton[30]. The *vacAs1m2* genotype is more common in our Iranian patients, as previously described in Iran[31]. As reviewed by Suzuki *et al*[32], the predominant *vacA* genotypes in Asia, Europe and Africa are *vacAs1m1* and their subtypes, which is in contrast to our genotypes in Iranian isolates.

In spite of the low frequency of *vacAs1m1* genotypes in our study, isolates which carried the *vacAs1m1* gene showed significant associations with severe active chronic

gastritis. In a review by Hosseini *et al*[33], they concluded that in contrast to *vacA*, there is no correlation between *cagA* genotype and disease status in the majority of studies conducted in Iran; but results of our study, however, proposed both of these genetic markers as useful indicators for predicting clinical outcomes in the studied population.

The meta-analysis by Shiota *et al*[8] confirmed the importance of the presence of *iceA* gene for peptic ulcer, although the significance was controversial. Such different results between the *iceA* allelic types and clinical disorders could be explained by the difference in geographic regions. In our study we found a significant relationship between $iceA_2$ genotype and clinical outcomes (severe active chronic gastritis), which was also observed by Caner *et al*[34] in Turkey. As Shiota *et al*[8] summarized in their meta-analysis, most of the studies showed no association between $iceA_1$ and *cagA* status, which is in concordance with our study. Interestingly, the prevalence of mixed genotype $iceA_1$ + $iceA_2$ (40.8%) in our study was higher than other studies which had detected this mixed genotype[35-37]. So this high prevalence with mixed genotypes makes it difficult to analyze potential relationships between the presence of each *iceA* allelic variant and clinical outcomes. *babA* genotype was frequently found in *H. pylori* strains in our study (95.8%); this was associated with severe active chronic gastritis. Although this genotype showed significant correlation with the existence of *cagA,* no significant correlation was observed with other virulence factors such as $vacA\ s_1/s_2$, $vacA\ m_1/m_2$ and $iceA_1/iceA_2$. Chomvarin *et al*[38] detected the *babA* gene in 92% (103/112) of Thai patients, which is almost similar to our results; while in another study conducted in Cuba the prevalence of *babA* gene was lower (82.3%)[39]. It is important to mention that this PCR based method for *babA* genotyping must be confirmed by immunoblotting. Actually isolates were scored as *babA*-gene positive if the PCR and/or Southern blot analysis yielded a positive result[9].

Regarding the combination of genotypes, we observed twenty different genotypes which showed vast diversities in the *H. pylori* isolates in our study. Interestingly there was not any significant association between these combined genotypes and clinical outcomes, except for $cagA^+/vacAs_1m_1/iceA_2/babA$+ genotype which showed significant association with severe active chronic gastritis.

Genotypes of *H. pylori*, especially *cagA* and *vacA*, are reported to be crucial factors determining the cure rates. So to select an *H. pylori* eradication regimen, we need to consider *H. pylori* genotypes[10]. *H. pylori* genotype distributions and their correlations with disease outcomes have shown geographical differences. In this regard, Yamaoka *et al*[7] reviewed that within East Asia, where the incidence of gastric cancer is high, that $vacA\ m_1$ genotype is dominant; whereas in southern parts where the gastric cancer incidence is low, the m_2 genotype, which we observed in our study, is predominant. Dabiri *et al*[31] showed that there was statistically no association between the *vacA*, *cagA* and *cagE* status and clinical outcomes in Iranian patients, and recommended that other different markers may be more useful for this analysis. In comparison, in the current study, genotyping on the basis of *cagA*, *babA*, *vacA* and *iceA* was considered as a useful tool for predicting the clinical outcomes. Therefore, analyzing the multiple virulence factors of *H. pylori* (*cagA*, *vacA*, *iceA* and *babA*) might enable us to predict the patient's clinical outcome among Iranian patients. This prediction could be more accurate when accompanied by the impacts of environmental factors and host genetic polymorphisms such as interleukin-1 receptor antagonist gene polymorphism[37]. Nowadays, concurrent genotyping of *H. pylori* virulence markers and host factors is becoming increasingly crucial in the prediction of the diseases outcomes[40].

In conclusion, our results show that most of the *H. pylori* isolates were highly virulent on the basis of the main clinically allelic variants in three or four virulence factors they carried. The Iranian isolates predominantly possessed different genotypes which showed vast diversities. Significant association of the noted genotypes with severe active chronic gastritis suggests that this genotyping panel is a suitable tool for detection of virulent *H. pylori* isolates that could provide valuable guidance for prediction of the clinical outcomes.

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COMMENTS

Background

Infection with *Helicobacter pylori* (*H. pylori*) causes diverse clinical outcomes such as persistent gastritis, peptic ulcers, mucosa associated lymphoid tissue lymphoma and gastric cancer. One of the reasons for these different clinical outcomes is genetic diversity of *H. pylori*; therefore determination of the pattern of *H. pylori* genotypes and its correlation with disease outcome, which shows geographic differences, is crucial.

Research frontiers

The *H. pylori* genotyping may have multiple roles including prediction of clinical outcomes, impact on the *H. pylori* infection therapy, tracking human migration, and recently, the prediction of progression of gastric preneoplastic lesions. Therefore genotyping of *H. pylori* can be a valuable and multifunctional tool in the clinical field.

Innovations and breakthroughs

In the majority of previous studies, the researchers were not able to detect any significant relationship between their genotyping panels and clinical outcomes for *H. pylori* infections. Most of these studies had used few genetic markers. In order to overcome this disadvantage, the authors have chosen greater numbers of *H. pylori* genetic markers for studying this association.

Applications

The genotyping panel which contains eight important genetic markers can serve as a useful tool for typing of *H. pylori* isolates and, to some extent, predict clinical outcomes.

Peer review

This is an epidemiological paper with statistical analysis, dealing with the important question of association between certain *H. pylori* genotypes and specific

pathologies, and with the problem of predictive value of *H. pylori* infection genotyping. In the submitted manuscript this issue is dissected in fine detail and uses quite extensive clinical material, thus providing novel and more reliable data.

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BRIEF ARTICLE

Polymorphism in the interleukin-17A promoter contributes to gastric cancer

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Abstract

AIM: To evaluate the contribution of the *G-197A* polymorphism in the interleukin-17 (IL-17) promoter region to gastric cancer risk in an Iranian population.

METHODS: We performed a case control study using samples from 161 individuals with gastric cancer and 171 healthy controls. For each individual, the *G-197A* genotype was determined by restriction fragment length polymorphism analysis of polymerase chain reaction-amplified fragments. Statistical analyses were performed to determine whether any demographic or behavioral factors, infection with *Helicobacter pylori* (*H. pylori*), or a particular *G-197A* genotype was associated with gastric cancer risk.

RESULTS: We found that the *G-197A* genotype was significantly associated with increased gastric cancer risk (P = 0.001). Patients who were homozygous (AA) at position -197 were 2.9 times more likely to develop disease (95%CI: 1.56-5.4; P = 0.001). Furthermore, logistic regression analysis revealed that the presence of a single A allele increased the risk of gastric cancer up to 1.7-fold (95%CI: 1.26-2.369; P = 0.001). This association was observed for early stage gastric adenocarcinomas only, and was not linked to *H. pylori* infection.

CONCLUSION: These results suggest that carrying one or more *G-197A* polymorphisms at position -197 in the IL-17 promoter region significantly increases gastric cancer risk in this patient population.

Key words: Gastric cancer; Interleukin-17A; Cancer; *Helicobacter pylori*

Core tip: There is currently a need for genetic markers to identify individuals at risk for developing gastric cancer. In this study, we describe one such marker, a *G-197A* polymorphism in the interleukin-17A (IL-17A) promoter. Within our study population, individuals who carry the *G-197A* polymorphism in the IL-17A promoter region may be at a significantly greater risk of developing gastric cancer. Importantly, the presence of this polymorphism alone was sufficient to increase risk of gastric cancer development.

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INTRODUCTION

Gastric cancer is one of the most common causes of cancer-related deaths worldwide. Despite an overall decrease in gastric cancer incidence in recent years, this disease is still responsible for over 700000 deaths per year[1,2], and represents a significant medical burden in many countries. In Northern Iran, gastric cancer has a major impact on public health due to the high morbidity and mortality associated with this disease. Indeed, several Iranian provinces, including Manzadaran, Semnan, Golestan, and the greater Tehran area, report age-standardized incidence rates for gastric cancer ranging from 25.4-49.1[3]. While these high incidence rates may be partially explained by the fact that a significant proportion of this population is also colonized by the carcinogenic bacterium *Helicobacter pylori* (*H. pylori*)[3,4], the fact that this region maintains a high rate of gastric cancer despite an intensive *H. pylori* eradication program suggests that there are other host genetic and environmental factors involved in gastric cancer development.

Over the last several years, many studies have identified a variety of environmental, behavioral, and host genetic factors that play a role in gastric carcinogenesis across many patient populations. Among these behavioral factors are smoking and a high salt diet[5-8], which have been shown to be particularly important for disease development in Northern Iran[6]. However, there is currently a lack of information regarding which host genetic factors may play a role in carcinogenesis in the Iranian patient population. Previous reports have identified a group of host immune factors that, when aberrantly expressed, can influence the development of gastric disease. Among these factors are the interleukin-1 (IL-1), IL-8, IL-10, and tumor necrosis factor-α (TNF-α) genes, where specific polymorphisms have been associated with gastric cancer risk[9,10]. Additionally, in some instances, this effect can be compounded when the polymorphism exists in an *H. pylori* colonized individual. It is hypothesized that these polymorphisms result in a pro-inflammatory gastric environment, which may prime the tissue for cancer development.

Another, more recently described, pro-inflammatory cytokine is IL-17A (IL-17). This cytokine is one of a larger group of IL-17 family ligands and is primarily produced from a subset of $CD4^+$ effector cells known as Th17 cells[11-13]. IL-17 is involved in both innate and adaptive immunity and can act on a variety of cell types[11,12]. Recently, reports have indicated that certain *IL-17* polymorphisms are associated with autoimmune disease such as rheumatoid arthritis, graft *vs* host disease[14], and inflammatory diseases such as ulcerative colitis[15], suggesting that aberrant expression of this cytokine may polarize the body toward a variety of disease states. In addition, a recent study indicated that *H. pylori*-mediated induction of IL-17 may impact disease progression[16]; collectively, these studies highlight the importance of levels of IL-17 in a variety of diseases.

One particular *IL-17* polymorphism (G-197A or rs22759133) has also been associated with certain types of gastric cancer in both Japanese and Chinese populations[17,18]. The guanine to adenine substitution at position -197 within the IL-17 promoter region is located in close proximity to 2 nuclear factors activated T cell binding motifs[19]. Because this region was shown to be required for IL-17 expression[19], it is believed that cells that harbor this mutation produce higher levels of IL-17, which in turn upregulates IL-17-mediated immune responses. This hypothesis is supported by the fact that various types of tumors express increased levels of IL-17[12], and patients with gastric cancer have a greater number of circulating IL-17-producing Th17 cells than healthy controls[20]. Taken together, these findings highlight the potential role of IL-17 in gastric cancer development.

Herein, we describe an epidemiologic study in which we investigate the role of the IL-17 G-197A promoter polymorphism in gastric cancer risk among individuals from Northern Iran, which is traditionally a poorly studied population. We found that within this patient population the *G-197A* polymorphism was significantly more frequent in gastric cancer patients compared with controls. This association was independent of *H. pylori* colonization status. These data indicate that the *IL-17 G-197A* polymorphism may be a good indicator for susceptibility to gastric cancer development in this patient population.

MATERIALS AND METHODS

Study participants

All aspects of the current study were approved by the Medical Research Ethics Committee at the Mandazaran University of Medical Sciences and conformed to the ethical guidelines set forth in the Declaration of Helsinki. Prior to enrollment, all patients were given an explanation of the nature of the study, and written informed consent was obtained from all individuals. Enrollees from the Mandazaran province of Iran were accepted after seeking treatment at Imam Teaching Hospital or Touba Polyclinic between April 2008 and November 2011. The diagnosis of gastric cancer cases were made based on gastric endoscopy, and cases were defined using the International Classification of Diseases for Oncology IX, Protocol 151 and Lauren criteria[21]. In order to simplify TNM staging[22], Stages I A and I B were grouped into "Stage I ", and Stages ⅢA and ⅢB were similarly combined into "Stage Ⅲ". We enrolled a total of 161 patients with gastric cancer (89 male, 72 female), with a mean age of 62.6 ± 12.4 years. One hundred seventy-one healthy controls (84 male and 87 female) were also enrolled, with a mean age of 60.8 ± 12.8 years. Subjects within the control group were matched to the case group with respect to age, sex, ethnic background, and geographic origin. Demographics and behavioral and epidemiological risk factors were self-reported by study participants using a written questionnaire. Cigarette smokers were defined

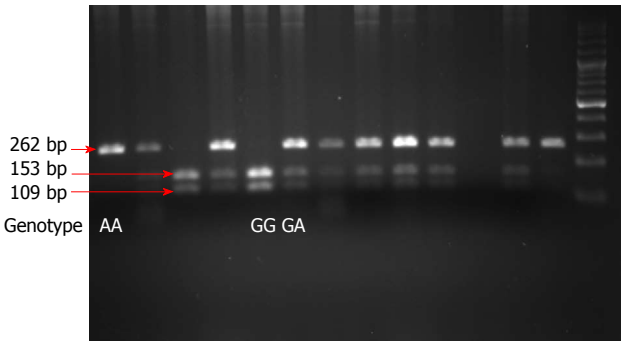

Figure 1 Interleukin-17 genotyping. A representative image of the results of an interleukin-17 (IL-17) genotyping assay is shown. The IL-17 promoters were amplified by polymerase chain reaction and the resulting products were digested with Xag I . Products were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The *IL-17 GG* genotype was evident as 153 and 109 bp fragments, GA as 262, 153 and 109 bp fragments, and AA as a single 262 bp fragment.

as those participants who reported smoking at least one cigarette per day for 12 mo. Consumption of salted fish, pickles, and fast food was defined as eating these items at least once a week for 6 mo.

H. pylori detection

All patients were tested for *H. pylori* infection using *H. pylori* specific IgG by ELISA (Diagnostic Automation, CA, United States) and by the urea breath test. Individuals that tested positive by either of these methods were considered as positive for *H. pylori*.

IL-17 genotyping

Venous blood collected from all study participants was used to isolate genomic DNA restriction fragment length polymorphism analysis of polymerase chain reaction-amplified fragments (PCR-RFLP) as previously described[18]. Briefly, each PCR amplification was performed using 1 μmol/L each of the forward (5'-AACAAGTAAGAATGAAAAGAGGACATGGT-3') and reverse (5'-CCCCCAATGAGGTCATAGAAGAATC-3') primers, 200 μmol/L of each dNTP, 2 mmol/L $MgCl_2$, 0.4 U of Hot Start Taq polymerase (Takara), 1X Takara Hot Start Taq PCR buffer, and 100 ng of genomic DNA in a final volume of 25 μL. Each reaction was initially denatured at 95 ℃ for 4 min, followed by 35 cycles of denaturation at 95 ℃ for 40 s, primer annealing at 60 ℃ for 35 s, and extension at 72 ℃ for 30 s, followed by a final extension at 72 ℃ for 4 min. Amplified PCR products were subjected to enzymatic digestion with XagI (Fermentas) for 12 h at 60 ℃ and visualized after separation by 3% sodium dodecyl sulfate polyacrylamide gel electrophoresis and staining with ethidium bromide. This procedure allowed us to clearly differentiate between the homozygous GG, heterozygous GA, and homozygous AA genotypes: the resulting restriction digest products from individuals with a homozygous (GG) genotype were 153 bp and 109 bp; digestion products from individuals who were heterozygous (GA) were 262 bp, 153 bp, and 109 bp; a single 262 bp product was produced from individuals with a homozygous (AA) genotype (Figure 1).

Table 1 Demographic data of the gastric cancer patients and healthy controls

	Gastric cancer (n = 161)	Control (n = 171)	P-value[1]
Age (yr)	62.56 ± 12.44	60.81 ± 12.76	0.21
Sex (M/F)	89/72	84/87	0.16
Marital status			
Single	8 (5)	3 (1.8)	
Married	149 (92.5)	166 (97.1)	0.002
Divorced	4 (2.5)	2 (1.2)	
Occupation			
Unemployed	4 (2.5)	1 (0.6)	0.003
Employed	24 (14.9)	35 (20.5)	
Housewife	48 (29.8)	75 (43.9)	
Other	85 (52.8)	60 (35.1)	
Education 12 yr	53 (32.9)	85 (49.7)	0.003

[1]Significance of categorical variables was assessed using the χ^2 test; Differences in age were evaluated using the Student t-test. Percentages are shown in parentheses. "Other" is defined as an occupation that does not fall into one of the defined groups.

Statistical analysis

After determining that all quantitative data were normally distributed (*via* Kolmograph-Smirnoph test), differences between patient populations were evaluated using the Student t-test. Qualitative differences between groups were assessed by the χ^2 test as indicated. The association between *IL-17* genotype and gastric cancer risk was determined using logistic regression analysis and an odds ratio (OR) with 95%CI. P-values $\leqslant 0.05$ were considered significant for all tests.

RESULTS

Patient demographics and epidemiology

The demographic data of gastric cancer patients and healthy controls are summarized in Table 1. Ages of study participants across the control group (n = 171) ranged from 24 to 87 years, and in the gastric cancer group (n = 161) from 28 to 86 years. The age difference between these 2 groups of participants was not statistically significant (P = 0.21). Similarly, the distribution of males and females in the study was also not significantly different between the gastric cancer group and the healthy controls (P = 0.16, χ^2 test). We did note a statistically significant difference in the distribution of married individuals in the cancer group and the healthy controls, where individuals in the gastric cancer group were more likely to be single or divorced (P = 0.002, χ^2 test). Similarly, we also noted that individuals within the gastric cancer group were more likely to be unemployed than those in the control group (P = 0.003, χ^2 test). Finally, we also detected a difference in the level of education between patients in the 2 groups; a significantly higher number of the healthy controls had > 12 years of education compared with the gastric cancer patients (P = 0.003, χ^2 test).

Table 2 Frequency of the distribution of the *G-197A* (rs2275913) polymorphism of the interleukin-17A gene in gastric cancer patients and healthy controls *n* (%)

Genotype	Cancer (*n* = 161)	Controls (*n* = 171)	OR	CI	*P*-value[1]
GG	56 (34.8)	78 (45.6)	1.00[2]		
GA	61 (37.9)	72 (42.1)	1.2	0.73-1.91	0.53
AA	44 (27.3)	21 (12.3)	2.92	1.56-5.4	0.001
G allele	173 (53.7)	228 (66.7)	1.00[2]		
A allele	149 (46.3)	114 (33.3)	1.72	1.26-2.36	0.001
A allele carriage (AA + GA *vs* GG)	105 (64.2)	93 (54.4)	1.57	1.01-2.45	0.04

Genotype frequencies are indicated in absolute values, with the percentage in parentheses. G allele and A allele indicates the total number of each individual allele within each group. [1]Two-sided χ^2-test; [2]The first allele or genotype is considered as the reference for this analysis. OR: Odds ratio.

Frequency and distribution of IL-17 genotypes and gastric cancer risk

We next evaluated the distribution of the IL-17-197 alleles between the 2 study groups essentially as previously described[17,18]. The genotype frequencies of this polymorphism in controls were within the Hardy-Weinberg equilibrium (P = 0.49). As shown in Table 2, the predominant genotype found in gastric cancer patients was the heterozygous GA allele (38%), followed by the homozygous alleles GG (35%) and AA (27%). In contrast, within the healthy control group, the most common genotype was the wildtype GG allele (45.6%) followed by the heterozygous GA allele (42%) and the homozygous AA allele (12%). While the difference in the distribution of the GG and GA genotypes between the gastric cancer and control groups was not statistically significant, the finding that a larger number of cancer patients carried the AA allele was significant (P = 0.001, χ^2 test). There was also a significant difference in the frequency of the A allele between the 2 groups; this allele was present in 46% of gastric cancer patients compared with only 33% of healthy controls (P = 0.001, χ^2 test). We next performed a multivariate regression analysis to determine the predictive value of the *G-197A* polymorphism for gastric cancer development. After correcting for covariates such as age, sex, and *H. pylori* infection, this analysis indicated that the presence of the A allele increased gastric cancer risk by 1.7-fold (95%CI: 1.26-2.36; P = 0.001). The presence of the AA mutant genotype increased the odds of developing gastric cancer up to 2.9-fold (95%CI: 1.56-5.4; P = 0.001), indicating that the presence of the AA genotype at this locus is significantly associated with increased gastric cancer risk. Additionally, harboring the allele (AA + GA) enhanced the risk of gastric cancer up to 1.6-fold (95%CI: 1.01-2.45; P = 0.04).

Effect of G-197A polymorphism and cancer progression

In order to determine whether the presence of the -197A allele was associated with disease progression within the gastric cancer group, we stratified a subset of the patients from this group based on TNM staging, extent of tumor cell differentiation, and the presence or absence of the mutant A allele (AA + GA *vs* GG); TNM information for 77 of the 161 patients enrolled in the gastric cancer group was available. A breakdown on tumor staging and degree of cellular differentiation are shown in Figure 2A and B, respectively. We placed individuals with lower grade malignancies (Stage Ⅰ or Ⅱ) in one group (n = 28), and those with Stage Ⅲ or Ⅳ malignancies into a second group (n = 49) (Table 3). Within the group with Stage Ⅰ or Ⅱ malignancies, 22 patients had at least one A allele (GA or AA genotype), while only 6 patients had the GG genotype. This difference in Stage Ⅰ / Ⅱ patients was statistically significant (P = 0.001, χ^2 test). Furthermore, the presence of the 197A allele increased the risk of gastric cancer development at the early stages of tumorigenesis by 6.3-fold (95%CI: 2.2-18.56; P = 0.001). In contrast, this association was not observed in patients with Stage Ⅲ/Stage Ⅳ malignancies or when we grouped the cancer patients by age, sex, *H. pylori* status, or tumor cell differentiation (Table 3). These data suggest

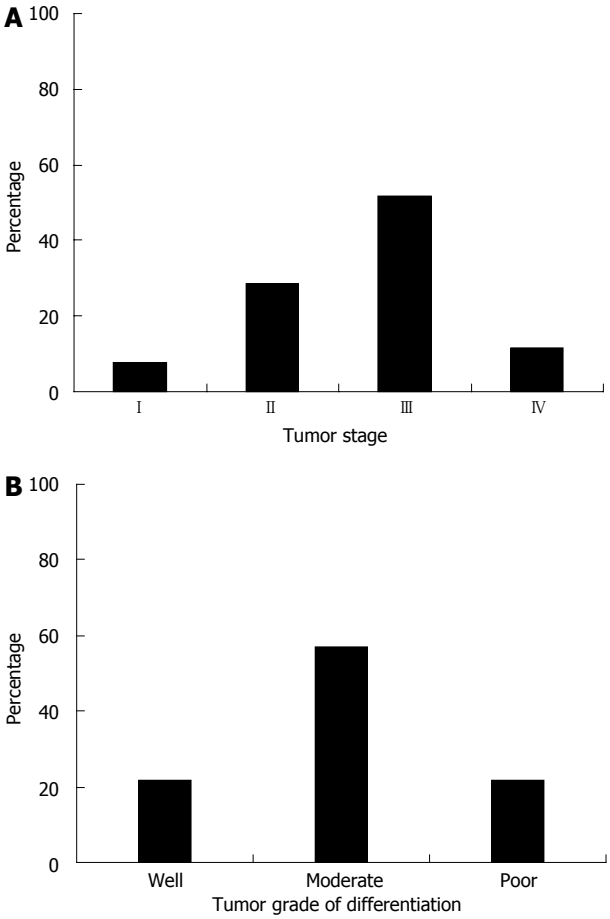

Figure 2 Tumor, node, metastasis staging, and cellular differentiation in the gastric cancer population. A: Breakdown of tumor staging among a subset of the gastric cancer population. Staging was categorized as described in the Materials and Methods section. For simplicity, individuals that were categorized as Stage ⅠA or ⅠB were grouped into Stage Ⅰ. Similarly, patients with Stage ⅢA or ⅢB tumors were grouped into Stage Ⅲ; B: The degree of cellular differentiation seen in patient tumors was graded as well, moderate, or poor as described in the Materials and Methods.

Table 3 Effect of *G-197A* polymorphism on gastric cancer development *n* (%)

	GA + AA	GG	OR	95%CI	*P*-value[2]
Age					
< 50 yr	18 (17.1)	4 (7.1)	2.7	0.8-8.4	0.09
≥ 50 yr	87 (82.9)	52 (92.9)			
Gender					
Male	51 (48.6)	21 (37.5)	1.6	0.8-3.05	0.19
Female	54 (51.4)	35 (62.5)			
H. pylori+	65 (61.9)	33 (58.9)	0.88	0.45-1.71	0.74
H. pylori-	40 (38.1)	23 (41.1)			
TNM stage[1]					
Ⅰ-Ⅱ	22 (55)	6 (16.2)	6.3	2.2-18.5	0.001
Ⅲ-Ⅳ	18 (45)	31 (83.8)			
Tumor differentiation					
Well	25 (23.8)	9 (16.1)	1.00[3]		
Moderate	57 (54.3)	35 (62.5)	0.56	0.24-1.34	0.19
Poor	23 (21.9)	12 (21.4)	0.66	0.23-1.86	0.43

[1]Data presented for 77 patients; [2]All comparisons between categorical variables were made using a two-sided χ^2 test; [3]Used as reference for tumor differentiation analyses. Values in parentheses indicate the percentage. *H. pylori*: *Helicobacter pylori*; TNM: Tumor, node, metastasis.

that while the presence of a mutant A allele at this locus increased the risk of developing a low grade (Stage Ⅰ or Ⅱ) malignancy, it was not a risk factor for progression to later stage cancer (Stage Ⅲ or Ⅳ).

DISCUSSION

Gastric cancer remains a significant source of morbidity and mortality worldwide. As such, being able to identify which patients or patient populations are most at risk for developing this severe disease is of the upmost importance. This fact is particularly true for geographical regions such as Iran that have exceptionally high disease rates[23]. Indeed, despite the alarmingly high rates of gastric cancer in this region, few studies have focused on the identification of host factors or mutations in these factors that may predispose members of this population to gastric cancer development. Once identified, these factors or mutations could then be exploited to aid in the diagnosis of high-risk patients.

Numerous studies have attempted to unravel the complex nature of gastric cancer development. From these studies it has become clear that carcinogenesis is a multi-factorial process that involves a combination of environmental/behavioral, and genetic factors. For many populations/geographic areas, including the focus of the current study, major environmental/behavioral risk factors for gastric cancer development have been identified[2,5-8]. Additionally, there have been many studies that have identified potential genetic markers or polymorphisms that are associated with gastric cancer risk. Several of these factors play a role in maintaining proper immune homeostasis, including the pro-inflammatory cytokines IL-1β, inducible nitric oxide synthase, TNF-α, IL-8, IL-10[9,10,24], and more recently IL-17[17,18]. However, since many of these factors have only been studied in limited patient populations, it remains unclear whether or not the prognostic value of these markers applies equally to all groups. In fact, as more studies are performed across a variety of patient populations, it has become evident that the degree to which these factors impact on disease development is often dependent upon the group being studied[9,25-28]. As a result, there is a need to examine the role of these factors in additional populations.

Here, we described a case-control study that examined the association of the G-197A IL-17 promoter mutation with gastric cancer development in an Iranian population. This particular polymorphism has been previously associated with an increased risk of gastric mucosal atrophy and gastric cancer in a Japanese population[17], as well as gastric cancer risk in a Chinese population[18]. In accordance with those studies, we found that the G-197A polymorphism is significantly associated with an increase in gastric cancer risk (Table 2). Specifically, harboring 2 copies of the mutant allele (AA) at this locus increased a patient's likelihood of developing cancer by a factor of 2.8 (Table 2). Furthermore, harboring only a single copy of this polymorphism (a heterozygous GA genotype) increased gastric cancer risk by 1.5 fold; this finding suggests that the effect of this polymorphism follows a dose-response. These data are consistent with the previous finding that the effect of the *G-197A* polymorphism on inflammation follows a similar dose-response pattern[17].

In healthy individuals, IL-17 is involved in both innate and adaptive arms of the immune system. Specifically, IL-17 is involved in induction of other pro-inflammatory cytokines as well as the recruitment and activation of inflammatory cells such as neutrophils and macrophages[11,29]. As the receptor for this cytokine is widely distributed on intestinal epithelial cells[12] and other tissue types[29], changes in the levels of IL-17 expression may have far reaching effects. This fact is illustrated by several studies, which have implicated increased IL-17 production with a variety of pathologic processes. Indeed, increased IL-17 transcript levels have been detected in patients with coronary artery disease[30], and inflammatory bowel disease[31], and specific *IL-17* polymorphisms have been associated with ulcerative colitis[15], rheumatoid arthritis[32], and graft *vs* host disease[14]. While these conditions may present quite differently from gastric cancer, the underlying commonality among these diseases is their inflammatory origin.

While the precise mechanistic role of the *G-197A* polymorphisms in gastric cancer development remains unclear, a plausible hypothesis is that increased/constitutive expression of IL-17 may skew the gastric environment to become pro-inflammatory. As chronic inflammation is a known precursor for gastric cancer development[33], this IL-17-mediated inflammatory environment may result in an increase in carcinogenic cellular damage, which predisposes an individual to develop disease. Once these initial steps have begun, the cancer may progress in an IL-17 dependent or independent manner. In the current study, we found that the -197A allele was

only significantly associated with the development of lower grade malignancies (Stage Ⅰ or Ⅱ) (Table 3). Similarly, a previous study linked the -197A polymorphism to an increased risk of poorly differentiated TNM Stage Ⅰ / Ⅱ cancer[18]. These data perhaps suggest that progression to more severe disease (Stages Ⅲ or Ⅳ) occurs at least partially in an IL-17-independent manner. However, as 18 of the 49 TNM Stage Ⅲ or Ⅳ cancer patients (Table 3) carried at least one mutant allele, we cannot completely rule out the possibility that this *IL-17* polymorphism impacts on disease progression to some extent.

Gastric cancer remains a global health problem. As diagnosis of this disease often occurs only after the progression to more severe stages, there is a serious need for diagnostic markers that could help preemptively screen patients in high-risk populations and identify those who are most at risk of developing disease. Because the *G-197A* polymorphism in the IL-17 promoter region is consistently linked to gastric cancer development in multiple populations, it may be a good global candidate marker to identify gastric cancer risk.

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COMMENTS

Background

Individuals who carry specific genetic polymorphisms can be prone to cancer development. As a result, these polymorphisms may be used to identify these at risk individuals. However, before a particular polymorphism can be reliably used as a marker for cancer risk, the link between the polymorphism and disease propensity must be verified in a multiple populations of people with diverse genetic backgrounds.

Research frontiers

Interleukin-17 (IL-17) is an important pro-inflammatory cytokine that is involved in both the innate and adaptive arms of the human immune system. One of the research hotspots in the field of IL-17 research is determining how increased or decreased levels of this cytokine effect human physiology and disease development.

Innovations and breakthroughs

Previous studies have identified the *G-197A IL-17* polymorphism as a potential genetic marker for gastric cancer risk. However, those studies were performed on a limited population that had a similar genetic background. The current study verified the *G-197A* polymorphism as a potential genetic marker for gastric cancer risk in a genetically distinct population. Their results reinforce the possibility of using this *IL-17* polymorphism as a marker for disease risk in many diverse populations.

Applications

The study highlights the possibility that the *IL-17 G-197A* polymorphism could be used as a marker for gastric cancer risk across diverse populations.

Terminology

A polymorphism is where multiple forms of a DNA sequence may be present at a single genetic site.

Peer review

The manuscript is quite well written. The results justify the conclusions drawn.

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BRIEF ARTICLE

Chronological changes in the liver after temporary partial portal venous occlusion

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Abstract

AIM: To investigate time-dependent changes caused by temporal portal vein obstruction and subsequent reperfusion in the lobe with or without an occluded portal vein.

METHODS: The portal vein (PV) of the anterior lobe of the liver of a male Wistar rat (8 wk-old) was obstructed (70%) for 12, 24, 36 and 48 h, respectively, and models were sacrificed at 48 h after reperfusion (each group: n = 10). The histological changes and the status of liver regeneration were compared between a liver biopsy performed on each lobe after temporary obstruction of the portal vein in the same rat liver, and the liver extracted at the time of sacrifice (48 h after reperfusion).

RESULTS: With regard to the obstructed lobe, the liver weight/body weight ratio significantly decreased according to obstruction time. On the other hand, in the non-obstructed lobe, there were no significant differences within each group. The duration of PV occlusion did not seem to be strong enough to introduce liver weight increase. Stimulation of liver regeneration was brought about in the non-occluded lobe by 12-h occlusion, and was sustained even at 48 h after reperfusion. The obstructed lobe atrophied with the passage of time in the obstructed state. However, the proliferating-cell nuclear antigen labeling index also increased at 48 h after reperfusion, and a repair mechanism was observed.

CONCLUSION: Temporary blood flow obstruction of the portal vein may become a significant trigger for liver regeneration, even with an obstruction of 12 h.

Key words: Temporary; Portal vein; Occlusion; Regeneration; Liver

Core tip: This paper describes the chronological effects of temporary portal venous branch ligation on liver regeneration in rats. These results imply that, in the future, it might be possible to control liver regeneration. In the clinical setting, we have just completely occluded the portal venous branch irreversibly.

Hamasaki K, Eguchi S, Soyama A, Hidaka M, Takatsuki M, Fujita F, Kanetaka K, Minami S, Kuroki T. Chronological changes in the liver after temporary partial portal venous occlusion. *World J Gastroenterol* 2013; 19(34): 5700-5705 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i34/5700.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i34.5700

INTRODUCTION

Permanent obstruction of the portal vein, as clinically

applied in portal branch ligation (PBL) or percutaneous transhepatic portal venous embolization, evokes liver regeneration[1-4]. This technique enables relatively major hepatic resection for malignancy in an occluded liver lobe[5-9]. In addition, PBL has been used to induce a regenerative stimulus for transplanted hepatocytes or pancreatic islet cells for cell therapy[10,11].

Although short term temporary occlusion can induce some degree of liver regeneration, an investigation of liver regeneration caused by temporary portal vein obstruction, as well as time-dependent changes resulting from reperfusion, has not yet been performed[12]. Therefore, the current study aimed to examine time-dependent changes in a lobe with a portal vein occlusion of an unobstructed portal vein caused by temporary portal vein obstruction and reperfusion as the central focus.

MATERIALS AND METHODS

Animals

Male Wistar rats (200-240 g, Japan SLC Inc., Shizuoka, Japan) were used for the experiments. All animals were maintained at 24 ℃ with a 12-h light-dark cycle and given free access to tap water and standard laboratory chow. The animals were treated in accordance with the guidelines stated in the University of Nagasaki Research Animal Resources during all experimental procedures.

Experimental design

The portal vein of the anterior lobe (medial and left lobes) of the liver of a male Wistar rat (8 wk old) was occluded (70%) for 12, 24, 36 and 48 h, respectively (Figure 1). Rats to be sacrificed were prepared at 48 h after each reperfusion (models for each group: n = 10, Figure 2). The histological changes over time and the status of liver regeneration were compared between liver biopsies performed from each lobe after temporary obstruction of the portal vein in the same rat liver, and the liver extracted at the time of sacrifice (48 h after reperfusion).

Liver to body weight ratio

The body weights and liver weights were recorded following the sacrifice of the rats to compare the rate of liver regeneration. The liver weight was expressed as a percentage of the body weight (%) and used as an index.

Histology and immunohistochemistry

Formalin-fixed paraffin embedded (4 μm) sections were used for hematoxylin-eosin (HE) staining. Proliferating-cell nuclear antigen (PCNA) immunostaining was performed to examine hepatocyte proliferation using a mouse monoclonal antibody against PCNA (clone-PC 10; Dako, Kyoto, Japan)[13]. Briefly, liver tissue specimens were fixed in 10% buffered formalin, embedded in paraffin and then cut into 5 μm sections. The deparaffinized sections were heated in a microwave three times in phosphate-buffered saline (PBS) for 5 min each and were then washed three times with PBS for 5 min each. After blocking endogenous peroxidase activity, the specimens

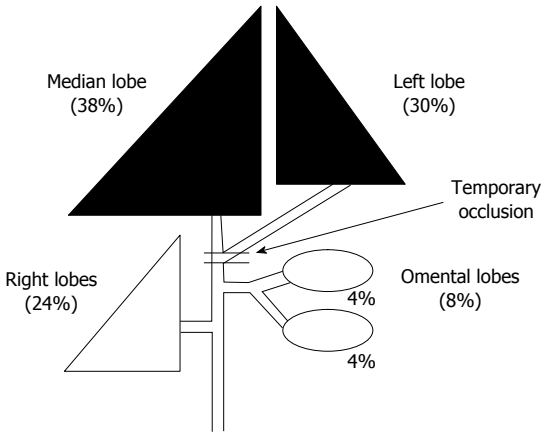

Figure 1 Schematic drawing of the rat model. The portal vein of the anterior lobe (black area) of the liver was occluded (68%) for various durations, while the arteries remained open.

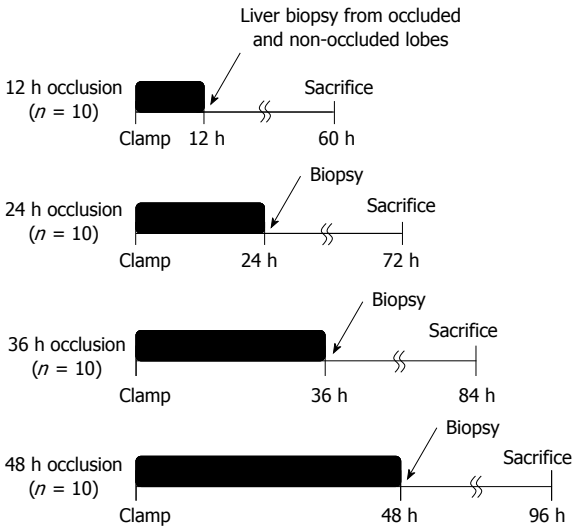

Figure 2 Experimental protocol. The portal vein of the anterior lobe (median and left liver lobes) of the liver of a male Wistar rat was occluded (70%) for 12, 24, 36 and 48 h. Rats to be sacrificed were prepared at 48 h after each reperfusion (models for each group: n = 10).

were washed three times with PBS for 5 min each. The sections were incubated with an antibody against PCNA overnight at 4 ℃. After washing several times with PBS, biotin-labeled secondary antibody was added for 1 h at room temperature. After washing several times with PBS, the tissue peroxidase activity was visualized using diaminobenzidine.

The PCNA labeling index (PCNA LI) was then determined as the number of PCNA-positive cells among 1000 counted cells.

Statistical analysis

All of the data were expressed as the mean ± SD. The Mann-Whitney U-test was used for data analysis. A level of $P < 0.05$ was considered statistically significant.

RESULTS

With regard to the obstructed lobe, the liver weight/body

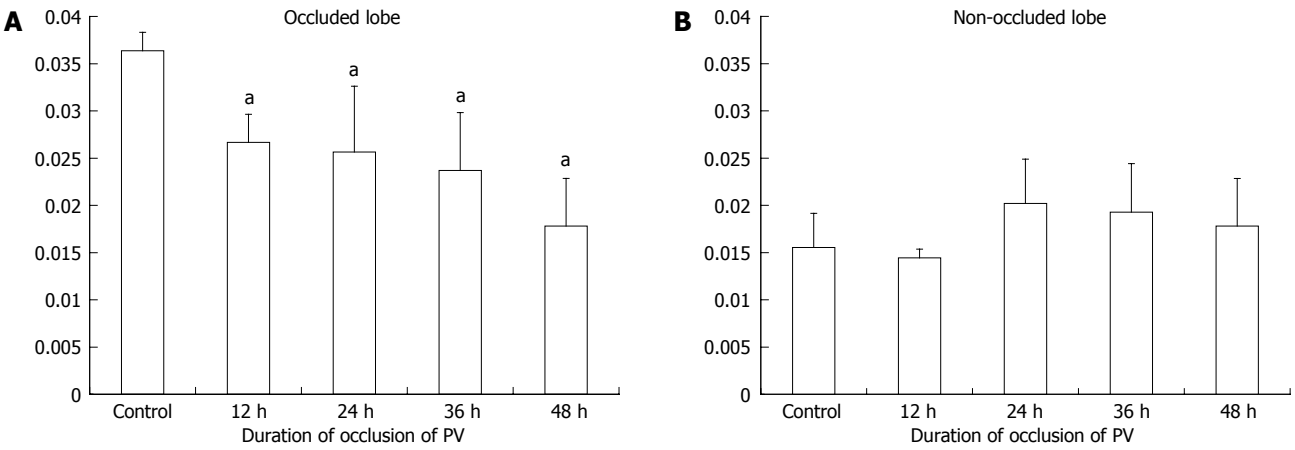

Figure 3 Liver weight/body weight. A: The liver weight/body weight ratio was decreased by temporary occlusion of the occluded anterior lobes; B: There were no significant differences within each group in non-occluded lobe. [a]*P* < 0.05 *vs* control group.

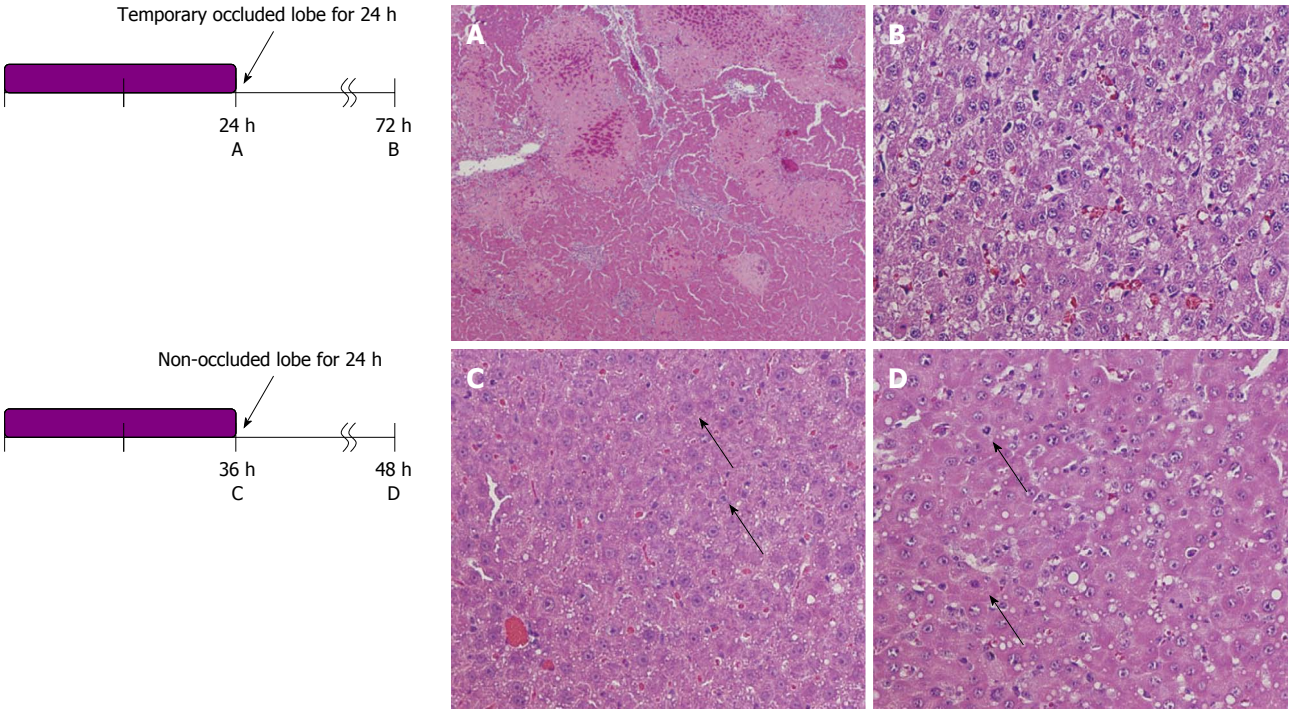

Figure 4 Changes in hepatic histology. Histology in temporary occluded lobes. A: 24 h occlusion, × 100; B: 36 h occlusion, × 400; C: In non-occluded lobes 24 h occlusion, × 100; D: 48 h occlusion, × 400 is shown by HE staining. In the occluded liver lobe, before reperfusion, coagulative necrosis may be observed around the central vein in proportion to the occlusion time. However, the above-mentioned necrotic area decreased at 48 h after portal vein reperfusion. In the non-occluded liver lobe, hepatocytes became hypertrophic, and some mitoses could be observed (arrows).

weight ratio significantly decreased with increasing obstruction time (Figure 3). On the other hand, in the non-obstructed lobe, there were no significant differences within each group. The duration of PV occlusion did not seem to be strong enough to induce an increased in liver weight.

Liver histology was investigated under HE staining (Figure 4). In the occluded liver lobe, before reperfusion, coagulative necrosis was observed around the central vein in proportion to the occlusion time. However, after 48 h of reperfusion, the above-mentioned necrotic area decreased. On the other hand, in the non-occluded liver lobe, hepatocytes became hypertrophic, and some mitoses were observed.

In the non-obstructed lobe, there were no significant differences in the PCNA LI within each group (Figure 5). LI seemed to peak at 36 h of biopsy (non-obstructed models at 12, 24, 36 and 48 h = 24%, 32%, 36% and 31%, Figure 5C). In the non-occluded lobe at 48 h after reperfusion (models for each group = 33%, 32%, 36% and 32%), the PCNA LI was still significantly increased at all points compared with the control (Figure 5D).

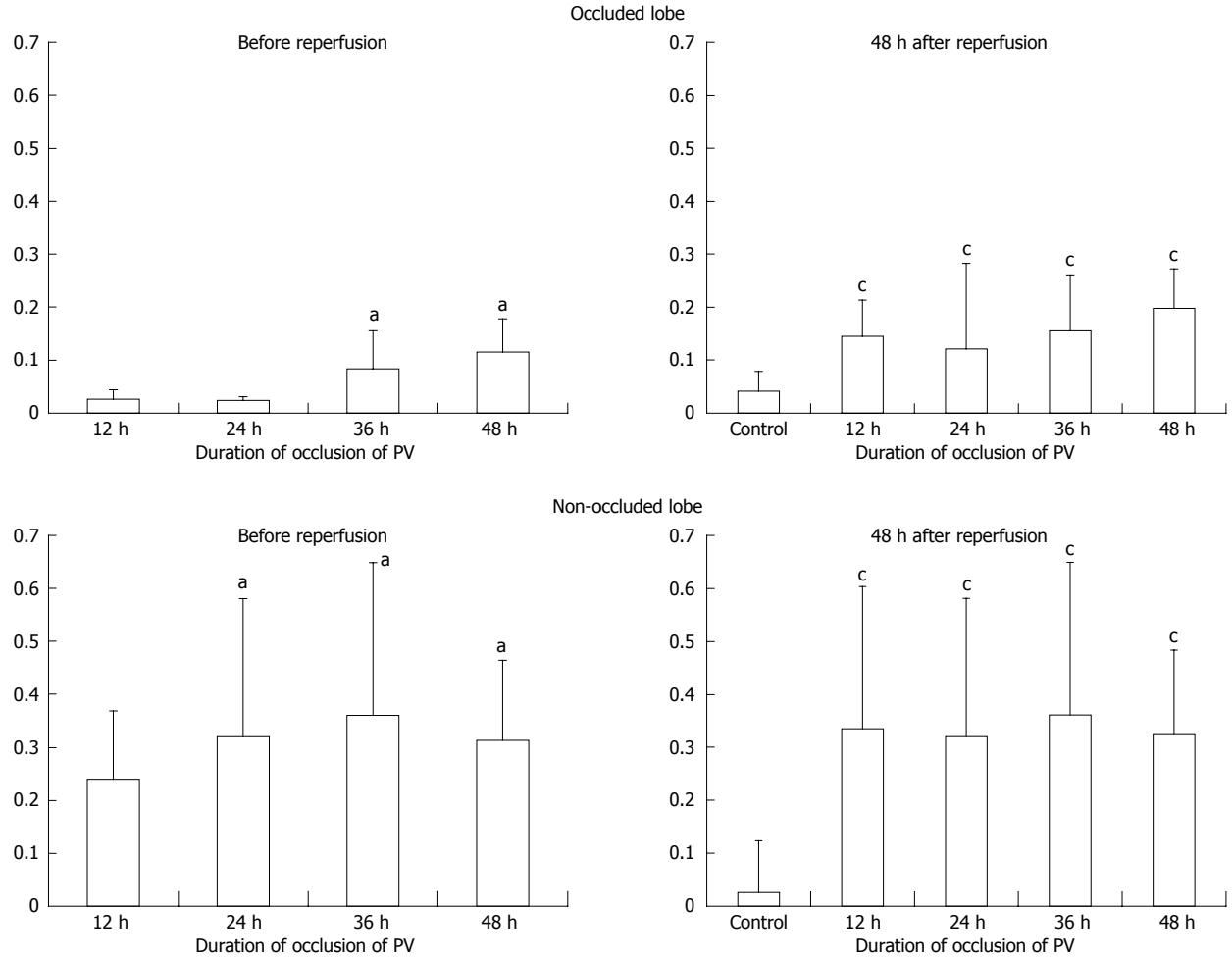

Figure 5 Proliferating-cell nuclear antigen labeling index. Proliferating-cell nuclear antigen (PCNA) labeling index in each lobe at the time before reperfusion and 48 h after reperfusion. [a]*P* < 0.05 *vs* 12 h group; [c]*P* < 0.05 *vs* 24 h group. PV: Portal vein.

On the other hand, hardly any positive cells were observed in a biopsy of the obstructed lobe (obstructed models at 12, 24, 36 and 48 h = 2%, 2%, 8% and 10%). However, at 48 h after reperfusion, an increase in LI was observed (models for each group = 14%, 12%, 15% and 19%).

DISCUSSION

Portal venous branch ligation or embolization (PBL or PBE) can induce atrophy of the ligated lobe, while inducing hypertrophy of a non-ligated lobe, which enables extended hepatectomy for a malignant tumor in a ligated lobe[14-16]. In addition, PBL has been used as a regenerative stimulator to induce transplanted cell proliferation in animal models for hepatocyte-based cell therapy[17-23]. The length of time of occlusion needed to induce remnant liver regeneration, *i.e.*, temporary portal venous occlusion, remains unknown. In the present study. the stimulation of liver regeneration brought about in the non-occluded liver lobe was sustained, even after 48 h from reperfusion. Thus, temporary blood flow obstruction of the portal vein may be a significant trigger of liver regeneration, with an obstruction of at least 12 h. However, the duration of PV occlusion in this study did not seem to be long enough to induce an increase in liver weight.

Interestingly, there was no significant difference in PCNA LI in the non-occluded lobe according to the duration of portal vein occlusion up to 48 h. Therefore, in the clinical setting, the same extent of liver hypertrophy may be induced with temporary balloon occlusion in as short as 12 h, to minimize an invasive procedure, although there might be difference among species.

As a cell therapy, many investigator have used liver for the engraftment of many cell types[10-12,14-16]. In fact, transplanted cells (hepatocytes, pancreatic islet cells or genetically engineered cells) could be induced to proliferate using temporary portal venous occlusion. Although there must be some differences between humans and rodents in terms of liver regenerative activity, our results provide a new insight into temporary stimulation of liver regeneration for subsequent treatment procedures[24-26].

On the other hand, the PCNA labeling index of the obstructed lobe was also increased 48 h after portal venous reperfusion. This could be a repair mechanism for portal vein ischemia in the occluded lobe, although PCNA LI was lower compared to that in the non-occluded lobe that undergoes liver regeneration[14]. Although it

was not observed up to 48 h, this result of the temporary occlusion of the lobe provided an interesting phenomena; however, the lack of temporal portal venous flow could become atrophic if portal venous ischemia had lasted longer. The duration for the "point of no return" should be investigated in further research.

In conclusion, a temporary blood flow obstruction of the portal vein may be a significant trigger for liver regeneration, even with an obstruction of 12 h. The histological changes in the unobstructed lobe and obstructed lobe in cases of temporary blood flow obstruction of the portal vein and at 48 h after reperfusion were described.

COMMENTS

Background

Permanent obstruction of the portal vein, as clinically applied in portal branch ligation (PBL) or percutaneous transhepatic portal venous embolization, evokes liver regeneration. This technique enables relatively major hepatic resection for malignancy in an occluded liver lobe. In addition, PBL has been used to induce a regenerative stimulus for transplanted hepatocytes or pancreatic islet cells for cell therapy.

Research frontiers

Portal venous branch ligation or embolization can induce atrophy of the ligated lobe, while inducing hypertrophy of a non-ligated lobe, which enables extended hepatectomy for a malignant tumor in a ligated lobe. In addition, PBL has been used as a regenerative stimulator to induce transplanted cell proliferation in animal models of hepatocyte based cell therapy. The length of time of occlusion required to induce remnant liver regeneration, *i.e.*, temporary portal venous occlusion, remains unknown.

Innovations and breakthroughs

The histological changes and the status of liver regeneration were compared between a liver biopsy performed on each lobe after temporary obstruction of the portal vein in the same rat liver, and the liver extracted at the time of sacrifice (48 h after reperfusion).

Peer review

This research is very important because it shows that a temporary obstruction of the portal vein as a trigger of liver regeneration. Recently, it has been used as a regenerative stimulator to induce transplanted cell proliferation. Thus, the manuscript approached an interesting subject from the surgical and scientific points of view; however, several aspects should be better evaluated.

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BRIEF ARTICLE

Efficacy of treatment with rebamipide for endoscopic submucosal dissection-induced ulcers

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Abstract

AIM: To prospectively compare the healing rates of endoscopic submucosal dissection (ESD)-induced ulcers treated with either a proton-pump inhibitor (PPI) or rebamipide.

METHODS: We examined 90 patients with early gastric cancer who had undergone ESD. All patients were administered an intravenous infusion of the PPI lansoprazole (20 mg) every 12 h for 2 d, followed by oral administration of lansoprazole (30 mg/d, 5 d). After 7-d treatment, the patients were randomly assigned to 2 groups and received either lansoprazole (30 mg/d orally, *n* = 45; PPI group) or rebamipide (300 mg orally, three times a day; *n* = 45; rebamipide group). At 4 and 8 wk after ESD, the ulcer outcomes in the 2 groups were compared.

RESULTS: No significant differences were noted in patient age, underlying disease, tumor location, *Helicobacter pylori* infection rate, or ESD-induced ulcer size between the 2 groups. At both 4 and 8 wk, the healing rates of ESD-induced ulcers were similar in the PPI-treated and the rebamipide-treated patients (4 wk: PPI, 27.2%; rebamipide, 33.3%; *P* = 0.5341; 8 wk: PPI, 90.9%; rebamipide, 93.3%; *P* = 0.6710). At 8 wk, the rates of granulation lesions following ulcer healing were significantly higher in the PPI-treated group (13.6%) than in the rebamipide-treated group (0.0%; *P* = 0.0103). Ulcer-related symptoms were similar in the 2 treatment groups at 8 wk. The medication cost of 8-wk treatment with the PPI was 10945 yen *vs* 4889 yen for rebamipide. No ulcer bleeding or complications due to the drugs were observed in either treatment group.

CONCLUSION: The healing rate of ESD-induced ulcers was similar with rebamipide or PPI treatment; however, rebamipide treatment is more cost-effective and prevents granulation lesions following ulcer healing.

Key words: Early gastric cancer; Rebamipide; Endoscopic submucosal dissection; Gastric ulcer; Proton-pump inhibitor

Core tip: In this prospective randomized, parallel-controlled study, we demonstrated that rebamipide monotherapy was as effective as proton-pump inhibitor (PPI) in the healing of endoscopic submucosal dissection-induced ulcers, regardless of the location of the resected cancer, the degree of atrophic gastritis, or the presence of *Helicobacter pylori* infection. In addition, rebamipide treatment is more cost-effective and results in a better quality of ulcer healing compared with the PPI lansoprazole.

Takayama M, Matsui S, Kawasaki M, Asakuma Y, Sakurai T, Kashida H, Kudo M. Efficacy of treatment with rebamipide for endoscopic submucosal dissection-induced ulcers. *World J Gastroenterol* 2013; 19(34): 5706-5712 Available from: URL:

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INTRODUCTION

Endoscopic mucosal resection (EMR) is a well-established curative treatment for gastric neoplasms, such as early gastric cancer confined to the mucosa. However, EMR, performed using conventional techniques such as strip biopsy or cap EMR, does not always achieve *en bloc* resection. Thus, endoscopic submucosal dissection (ESD) has become the preferred treatment method. Compared with EMR, ESD facilitates the collection of larger specimens, regardless of lesion size or location, resulting in a higher rate of *en bloc* and histologically complete resection. Moreover, the rate of local recurrence of tumors after ESD may be lower than that after conventional EMR[1]. However, the iatrogenic ulcer that develops as a result of ESD is large, and requires a considerably longer healing time compared to that resulting from conventional EMR.

Proton-pump inhibitors (PPIs) are the most effective medications for the treatment of ESD-induced ulcers. However, studies have shown that PPI monotherapy does not heal the ESD-induced ulcers sufficiently within 4 wk[2-6]. Increased understanding of the mucosal defense system has prompted the development of mucoprotective agents for clinical use in Japan. The efficacy of combination treatment involving PPIs and the mucoprotective agent rebamipide in the early treatment of ESD-induced ulcers and in the prevention of relapse of such disorders has been clearly indicated[2-5].

Rebamipide [2-(4-chlorobenzoylamino)-3-[2-(1H)-quinolinon-4-yl]-propionic acid), a novel mucosal-protective and ulcer-healing drug, is widely prescribed in East Asia. Previous studies have indicated that rebamipide is effective in the treatment of gastric ulcers as well as decreasing the recurrence rate, without affecting the *Helicobacter pylori* infection status of the patients[7-12]. In addition, previous randomized-controlled studies have also found that rebamipide can prevent the formation of peptic ulcers induced by the administration of nonsteroidal anti-inflammatory drugs (NSAIDs) and can suppress the mucosal inflammation associated with chronic erosive gastritis[13,14]. However, to our knowledge, no reports on the use of rebamipide for the treatment of ESD-induced ulcers have been published.

In the present study, we have prospectively evaluated the efficacy of rebamipide monotherapy in comparison to PPI monotherapy for the treatment of iatrogenic ulcers resulting from ESD for early gastric cancers.

MATERIALS AND METHODS

Patients

We examined 90 consecutive patients with early gastric cancer who had been treated with ESD at Kinki University Hospital between February 2011 and January 2013. The study protocol was approved by the Kinki University Ethics Committee, and all participants provided written informed consent before undergoing ESD. In addition, the study was registered at the University Hospital Medical Information Network 000005134. All patients with early gastric cancer, including well-differentiated or moderately differentiated adenocarcinoma, were included in the study. The exclusion criteria were as follows: (1) current use of other anti-ulcer drugs, aspirin, NSAIDs, or prednisolone; (2) treatment with anti-coagulative agents; or (3) previous endoscopic treatment or surgery.

ESD procedure

ESD was performed with an insulation-tipped knife (KD-610L; Olympus Medical Systems, Tokyo, Japan) and a flush knife (BTDK2618JB; Fujifilm Medical System, Tokyo, Japan). The electrosurgical unit used was A VIO-300D (ERBE). The injection solutions contained glycerin with 1% indigo carmine dye and, depending on the tumor location, hyaluronic acid sodium (0.4%) was also added. The ulcers that developed after ESD were carefully examined endoscopically, and any visible vessels were heat-coagulated by using hot biopsy forceps (KD-410LR; Olympus Medical Systems). Thereafter, the resected specimens were stretched, pinned flat on a rubber plate, and measured.

Study design

The design of this single-center, open-label, prospective, randomized, parallel-controlled study is illustrated in Figure 1.

After ESD, all patients were administered an intravenous infusion of lansoprazole (20 mg; Takepron; Takeda Pharmaceutical, Osaka, Japan) every 12 h for 2 d, and received oral lansoprazole (30 mg/d) for 5 d. On postoperative day 7, the patients were randomly assigned to 2 groups and received either lansoprazole at a dose of 30 mg/d orally (PPI group; $n = 45$), or rebamipide (Mucosta; Otsuka Pharmaceutical Co., Tokyo, Japan) at a dose of 300 mg orally, 3 times a day ($n = 45$) for 8 wk. The primary endpoint was endoscopically documented ulcer healing; complete healing was defined as regression to the S-stage on the Sakita and Miwa scale[23]. Moreover, we evaluated the healing rates of atrophic gastritis based on the Kimura and Takemoto classification[24], in the presence or absence of *Helicobacter pylori* (*H. pylori*) infection. We also compared the response of ulcers in relation to their locations in the stomach (lower, middle, or upper).

The secondary endpoint was the ulcer reduction ratio, which was compared according to the ulcer location. For calculation of the ratio, we determined the maximum diameters of the ulcers and the diameters perpendicular to the maximum diameters, which were measured using a bendable endoscopic measuring device (M2-3; Olympus Corp., Tokyo, Japan). Moreover, we determined the ulcer size (maximal diameter × diameter perpendicular to the maximal diameter). At 4 and 8 wk after ESD, the healing and reduction rates for the ulcers were compared between the 2 groups. In addition, at 8 wk after ESD, we

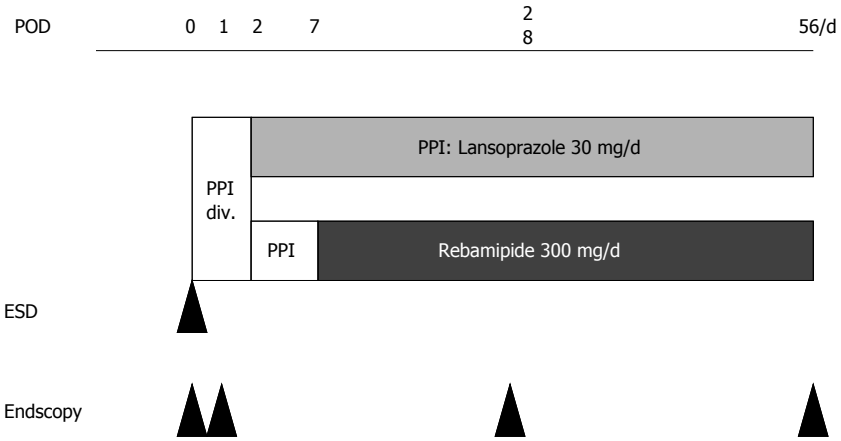

Figure 1 Study design. After ESD, all patients were administered an intravenous infusion of lansoprazole (20 mg; Takepron; Takeda Pharmaceutical, Osaka, Japan) every 12 h for 2 d, and received oral lansoprazole (30 mg/d) for 5 d. On postoperative day 7, the patients were randomly assigned to 2 groups and received either lansoprazole at a dose of 30 mg/d orally (PPI group; n = 45), or rebamipide (Mucosta; Otsuka Pharmaceutical Co., Tokyo, Japan) at a dose of 300 mg orally, 3 times a day (n = 45) for 8 wk. ESD: Endoscopic submucosal dissection; PPIs: Proton-pump inhibitors; div.: Drip intravenous infusion; POD: Postoperative days.

Table 1 Patient characteristics

	Lansoprazole group (n = 45)	Rebamipide group (n = 45)	P-value
Age (yr) (mean ± SD, median)	70 ± 7.8, 72	67 ± 8.0, 67	0.0930
Gender (M/F)	36/9	31/14	0.2269
H. pylori-infection	86.7%	84.4%	0.7643
Smoker	31.1%	33.3%	0.8215
Drinking alcohol	46.7%	42.2%	0.6714
History of disease	46.7%	31.1%	0.1301
Complicated disease	71.1%	71.1%	1.0000
Location of tumors			0.1620
Low	23	16	
Middle	17	26	
Upper	5	3	
Tumor size			0.4986
> 20 (mm)	13	16	
≤ 20 (mm)	32	29	
Histologic classification			0.6939
Tub1	41	42	
Tub2	4	3	
Dissected size (mean ± SD, mm)	30.5 ± 7.8	30.6 ± 6.4	0.9413
Dissected area (mean ± SD, mm^2)	687.9 ± 393.1.7	712.3 ± 298.6	0.7417
Glandular atrophy			0.3167
C-1	0	0	
C-2	3	2	
C-3	11	14	
O-1	19	13	
O-2	12	13	
O-3	0	3	
CandO			0.6547
C	14	16	
O	31	29	
Intestinal metaplasia	71.1%	68.9%	0.8181

H. pylori: *Helicobacter pylori*; M/F: Male/female.

evaluated the scar status of the ESD-induced ulcers according to the Quality of Ulcer Healing (QOUH).

Statistical analysis

Patient baseline characteristics and ulcer reduction ratios were compared using Pearson's χ^2 test or Student's t-test. Pearson's χ^2 test was also used to compare the healing rates of the ESD-induced ulcers and for the evaluation of the scar status of the ESD-induced ulcers according to the QOUH. Statistical significance was defined as $P < 0.05$.

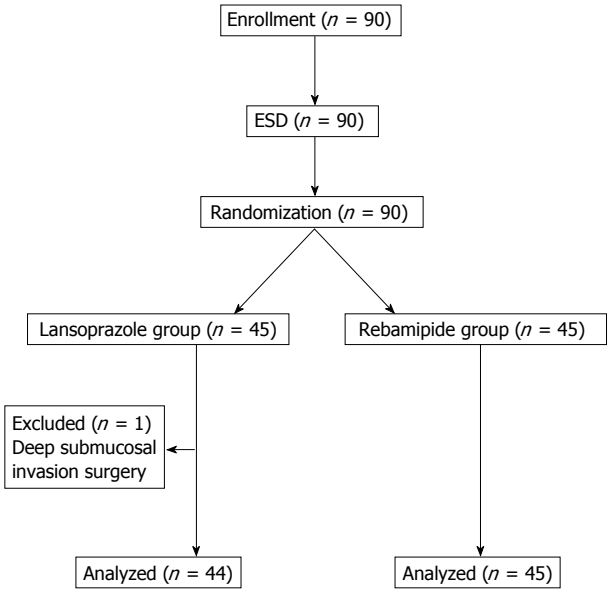

Figure 2 Flow chart of study participants. ESD: Endoscopic submucosal dissection.

RESULTS

Table 1 shows the patient characteristics of the 2 treatment groups. No significant differences were noted between the groups with regard to age; gender; tumor location; tumor size; histologic classification; ESD-induced ulcer size; glandular atrophy; history of disease; and the rates of *H. pylori* infection, smoking, drinking alcohol, or the presence of complicated disease or intestinal metaplasia. One patient was excluded from the PPI group because histologic examination of the resected specimen

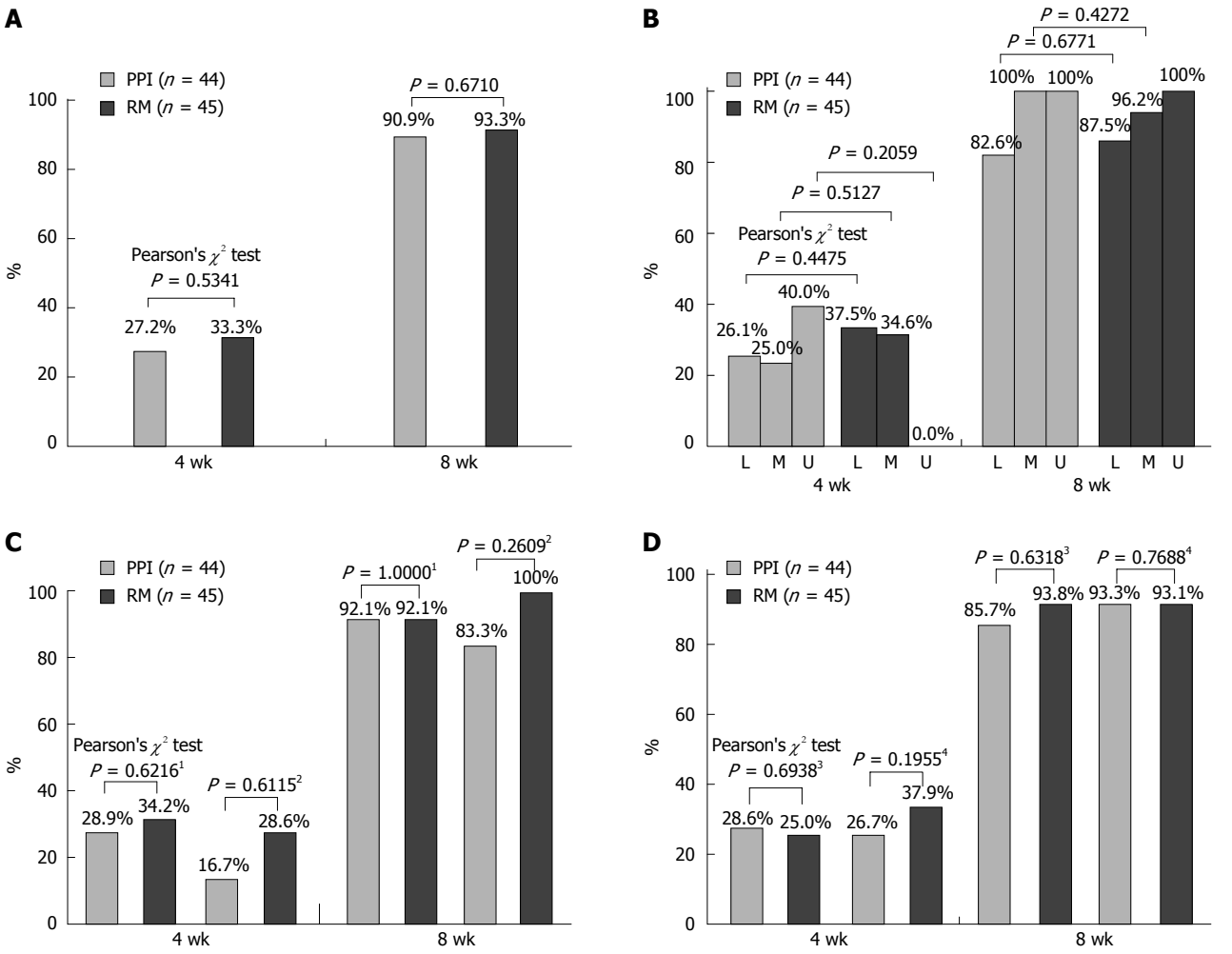

Figure 3 Rates of healing in both groups. A: The rates of healing to S stage in both groups; B: The rates of healing to S stage in both groups according to resected location. L: Low stomach, M: Middle stomach; U: Upper stomach. C: The rates of healing to S stage in both groups. [1]*Helicobacter pylori* (*H. pylori*) positive [2]*H. pylori* negative. D: The rates of healing to S stage in both groups in atrophic gastritis. [3]Closed type; [4]Open type. RM: Rebamipide.

indicated deep submucosal invasion (depth ⩾ 500 μm; SM2 invasion). Hence, 44 patients in the PPI group and 45 in the rebamipide group constituted the final study cohort (Figure 2).

Ulcer responses

The rates of ulcer healing (regression to S-stage) were not significantly different between the PPI group (27.2%) and the rebamipide group (33.3%) at 4 wk ($P = 0.5341$) or at 8 wk (90.9% for the PPI group and 93.3% for the rebamipide group; $P = 0.6710$) (Figure 3A). Moreover, at 4 and 8 wk, the healing rates were not significantly different between the treatment groups with regard to ulcer location (low, middle, or upper stomach; Figure 3B) or with regard to the presence of absence of *H. pylori* infection (Figure 3C). In addition, at 4 and 8 wk, the healing rates of atrophic gastritis (closed or open type) were similar in the 2 treatment groups (Figure 3D).

Reduction ratios of ESD-induced ulcers

The reduction ratios of ESD-induced ulcers were similar at 4 and 8 wk in the rebamipide group (98.0% and 99.9%, respectively) and in the PPI group (97.2% and 99.9%, respectively) (Figure 4). These ratios were not influenced by the locations of the ulcers in the stomach (low, middle, and upper).

Quality of ulcer healing and adverse events

Six patients in the PPI group developed unusual gastric lesions, which comprised an overgrowth of granulation tissue at the ulcer site. At 8 wk, the proportion of patients who developed a flat scar in the rebamipide group (100%) was found to be significantly higher than that in the PPI group (86.3%; $P = 0.0103$) (Figure 5). No ulcer bleeding or complications related to the drugs used after ESD were observed in any of the study subjects.

DISCUSSION

Some authors have reported that the combination therapy involving PPI and rebamipide is superior to PPI monotherapy in the healing of ESD-induced ulcers[2-5]; however, to our knowledge, no reports on the efficacy of rebamipide for the treatment of ESD-induced ulcers

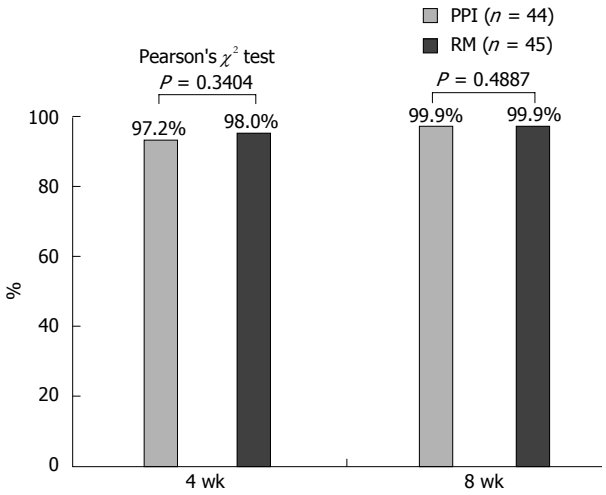

Figure 4 Reduction ratio of the endoscopic submucosal dissection-induced ulcers in both groups. PPI: Proton-pump inhibitor; RM: Rebamipide.

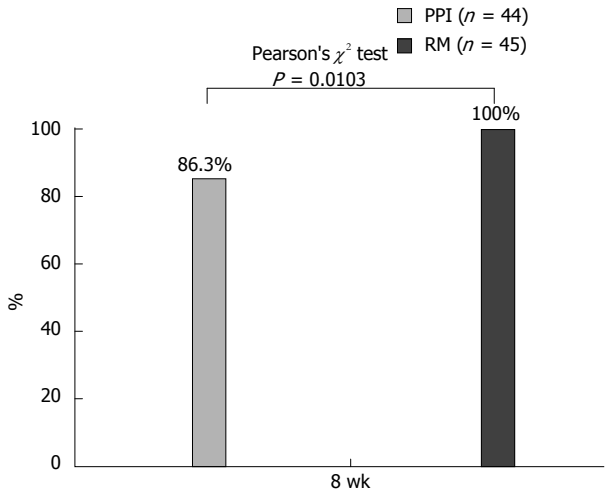

Figure 5 Proportion of patients who developed a flat scar after ulcer healing in both groups. PPI: Proton-pump inhibitor; RM: Rebamipide.

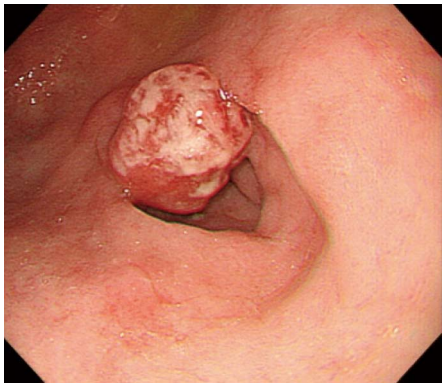

Figure 6 At 8 wk, granulation lesions following ulcer healing in the proton-pump inhibitors treated group.

have been published. In this prospective randomized, parallel-controlled study, we demonstrated that rebamipide monotherapy was as effective as PPI in the healing of ESD-induced ulcers, regardless of the location of the resected cancer, the degree of atrophic gastritis, or the presence of *H. pylori* infection.

Although the response of post-ESD ulcers to PPIs and rebamipide may be similar, the mechanisms of action of these drugs are different. PPIs decrease gastric acid production, whereas rebamipide stimulates the production of prostaglandins[19], epidermal growth factor[12,20], and nitric oxide[21], and decreases the level of oxygen-free radicals[22]. These mucosal protective actions of rebamipide appear to promote ulcer healing. Fujiwara *et al*[3] showed that 8 wk of PPI and rebamipide treatment was particularly effective for patients with severe atrophic gastritis, classified as O-3. Severe atrophic gastritis may result in the formation of a low-acid environment in the stomach; therefore, acid-suppressive agents such as PPI alone may have a limited effect. However, rebamipide can be effective in this environment because of its different mechanism of action. We believe that this is a contributing factor to the similar efficacies observed between PPIs and rebamipide regardless of the degree of atrophic gastritis.

Previous studies have reported that various mechanisms are involved in the effects of rebamipide on *H. pylori*-positive atrophic gastritis; these include prevention of adhesion of the bacteria to gastric epithelial cells, and inhibition of *H. pylori*-induced secretion of prostaglandin E2 from neutrophils and interleukin-8 expression in gastric epithelial cells[25-28]. Terano *et al*[15] indicated that the treatment of gastric ulcers with rebamipide promotes ulcer healing regardless of the success or failure of *H. pylori* eradication therapy, and Higuchi et al showed that rebamipide prevents the recurrence of gastric ulcers without affecting the *H. pylori* infection status[10]. In the present study, PPI and rebamipide appeared to aid in ulcer healing without affecting the *H. pylori* infection status.

Moreover, we noted that the proportion of patients who developed a flat scar at the ulcer site was significantly higher in the rebamipide group than in the PPI group. Thus, rebamipide appears to be more effective than PPIs in improving the QOUH. In animal studies, rebamipide was found to improve the QOUH by increasing the level of prostaglandin E_2 and decreasing the levels of malondialdehyde and interleukin-8 in the gastric mucosa[16]. In the present study, the unusual elevated gastric lesions that were observed following ulcer healing could not be easily characterized as benign granulation tissue or a malignant recurrence without performing a biopsy (Figure 6). Therefore, we believe that improvement in QOUH is essential for preventing the occurrence of mucosal protrusion due to the growth of granulation tissue.

The most frequent complication that occurs after endoscopic therapy is bleeding, and the rate of intraoperative bleeding is significantly higher with ESD than with EMR. Jeong *et al*[17] reported that PPIs may be more effective than histamine H_2 inhibitors in preventing bleeding after ESD by promoting a more rapid healing of these large iatrogenic ulcers[17]. Moreover, Uedo *et al*[18] indicated that therapy with PPI was more effective than treatment

with histamine H_2 inhibitors in preventing delayed bleeding from ulcers induced by ESD. However, in the present study, no post-ESD bleeding or complications related to the drugs used were noted in the patients receiving rebamipide or PPI treatment; moreover, the ratio of ulcer reduction was at least 90% in both groups at 8 wk after initiation of therapy. Thus, our findings indicated that the rate of intraoperative bleeding was not significantly different between both the groups.

In addition, in the present study, we found that treatment with rebamipide was more cost-effective than treatment with the PPI lansoprazole. The cost of the 56-d treatment course was 4889 yen for rebamipide and 10945 yen for lansoprazole, which is a difference of 44.7%. This high difference in cost may be a factor in determining which medication to prescribe in the treatment of ESD-induced ulcers.

In conclusion, rebamipide monotherapy was equivalent to treatment with a PPI (lansoprazole) in the healing of ulcers induced by ESD for early gastric cancer. The similarity in the treatment efficacy was observed irrespective of the presence of *H. pylori* infection, the severity of atrophic gastritis, or the locations of the ulcers in the stomach. However, rebamipide therapy also resulted in a more favorable QOUH compared with that obtained by PPI treatment. Moreover, the treatment involving rebamipide was more cost-effective compared to the treatment with the PPI lansoprazole for the treatment of ESD-induced ulcers.

ACKNOWLEDGMENTS

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COMMENTS

Background

Endoscopic submucosal dissection (ESD) is useful for treating early gastric cancer. The artificial ulcer that is generated after ESD is large, and needs a considerably longer healing time compared with conventional endoscopic mucosal resection (EMR). Rebamipide is one of the mucoprotective antiulcer drug, and is widely employed treatment of gastric ulcer in Japan.

Research frontiers

Previous studies have shown that the combination therapy involving proton pump inhibitor (PPI) and rebamipide is superior to PPI monotherapy in the healing of ESD-induced ulcers; however, to people knowledge, no reports on the efficacy of rebamipide for the treatment of ESD-induced ulcers have been published. Therefore, the authors prospectively investigated differences in healing of ESD-induced ulcers according to treatment with PPI or rebamipide only.

Innovations and breakthroughs

In this prospective randomized, parallel-controlled study, the authors demonstrated that rebamipide monotherapy was as effective as PPI in the healing of endoscopic submucosal dissection-induced ulcers. In addition, rebamipide treatment was more cost-effective and resulted in a better quality of ulcer healing compared to the PPI lansoprazole treatment.

Applications

This article suggests that the healing rate of ESD-induced ulcers was similar with rebamipide or PPI treatment; however, rebamipide treatment is more cost-effective and prevents granulation lesions following ulcer healing. However, it is a small study, therefore, a prospective multicenter study with a large sample size should be performed to assess the efficacy of treatment with rebamipide for endoscopic submucosal dissection-induced ulcers.

Terminology

ESD is a well-established curative treatment for early gastric cancer. ESD facilitates the collection of larger specimens, regardless of lesion size or location, resulting in a higher rate of en bloc and histologically complete resection. However, the iatrogenic ulcer that develops as a result of ESD is large, and requires a considerably longer healing time compared to that resulting from conventional EMR. Rebamipide is a novel mucosal-protective and ulcer-healing drug that has been widely prescribed in East Asia. Rebamipide stimulates the production of prostaglandins, epidermal growth factor, and nitric oxide, and decreases the level of oxygen-free radicals. These mucosal protective actions of rebamipide appear to promote ulcer healing.

Peer review

This paper is well written. The clinical results are appropriately described. The authors present the Efficacy of treatment with rebamipide for endoscopic submucosal dissection-induced ulcers. The data indicate the healing rate of ESD-induced ulcers was similar with rebamipide or PPI treatment; however, rebamipide treatment is more cost-effective and prevents granulation lesions following ulcer healing.

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BRIEF ARTICLE

Comparison of pancreatic acinar cell carcinoma and adenocarcinoma using multidetector-row computed tomography

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Abstract

AIM: To distinguish acinar cell carcinoma (ACC) from pancreatic adenocarcinoma (AC) by comparing their computed tomography findings.

METHODS: Patients with ACC and AC were identified on the basis of results obtained using surgically resected pancreatectomy specimens. The preoperative computer tomographic images of 6 acinar cell carcinoma patients and 67 pancreatic adenocarcinoma patients in 4 phases (non-contrast, arterial, portal venous, and delayed phase) were compared. The scan delay times were 40, 70, and 120 s for each contrast-enhanced phase. The visual pattern, tomographic attenuation value, and time attenuation curve were assessed and compared between AC and ACC cases using the χ^2 test, Wilcoxon signed-rank test, and Mann Whitney *U* test.

RESULTS: The adenocarcinomas tended to be hypodense in all 4 phases. The acinar cell carcinomas also tended to be hypodense in the 3 contrast-enhanced phases, although their computed tomographic attenuation values were higher. Further, 5 of the 6 acinar cell carcinomas (83%) were isodense in the non-contrast phase. The time attenuation curve of the adenocarcinomas showed a gradual increase through the 4 phases, and all adenocarcinomas showed peak enhancement during the delayed phase. The time attenuation curve of the acinar cell carcinomas showed peak enhancement during the portal venous phase in 4 cases and during the arterial phase in 2 cases. None of the 6 acinar cell carcinomas showed peak enhancement during the delayed phase.

CONCLUSION: The tumor density in the non-contrast phase and time attenuation curve pattern clearly differ between acinar cell carcinomas and adenocarcinomas, and multidetector-row computed tomography can thus distinguish these tumors.

Key words: Pancreatic acinar cell carcinoma; Pancreatic adenocarcinoma; Multidetector-row computed tomography; Visual pattern; Time attenuation curve

Core tip: The tumor density in the non-contrast phase and time attenuation curve pattern clearly differ between acinar cell carcinoma and adenocarcinomas, although both tumors tend to be hypodense in the contrast-enhanced phases.

Sumiyoshi T, Shima Y, Okabayashi T, Kozuki A, Nakamura T. Comparison of pancreatic acinar cell carcinoma and adenocarcinoma using multidetector-row computed tomography. *World J Gastroenterol* 2013; 19(34): 5713-5719 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i34/5713.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i34.5713

INTRODUCTION

Acinar cell carcinoma (ACC) is a rare malignant epithelial neoplasm that exhibits exocrine enzyme production, and it accounts for approximately 1% of all pancreatic neoplasms[1]. ACCs have been reported to be bulky tumors that mainly occur in the pancreatic head[2], and recent reports have shown that ACCs are often accompanied by intratumoral necrosis and have various specific extraparenchymal progression patterns, such as intraductal tumor growth (ITG) and venous tumor thrombus (VTT)[3-9]. Several reports have described the computed tomography (CT) findings of ACC: it is typically solitary and is accompanied by an intratumoral hypodense area when large. In terms of the visual pattern, although a few hyperdense ACCs have been reported, most ACCs have been reported to be hypodense on contrast-enhanced CT[10,11]. Despite these previous reports on imaging findings, the correct preoperative diagnosis of ACC remains difficult, and ACC is often misdiagnosed as another hypodense pancreatic tumor, namely, adenocarcinoma (AC)[11].

ACCs had been previously considered equally aggressive as ACs[12,13], and pretreatment differentiation between ACC and AC was not considered important. However, in recent years, increasing evidence has shown that ACCs are characterized by less aggressive growth and that ACC shows significantly better long-term survival than AC[12]. Further, although no consensus has been reached on surgery for metastatic ACCs, a few reports have described a good prognosis after resection of limited metastatic disease. Because the malignant potential of ACC and AC is significantly different, correct pretreatment distinction between these two tumors is very important.

This study aims to elucidate the characteristic CT findings of ACC to allow accurate diagnosis of even small ACCs. The visual pattern, CT attenuation value, and time attenuation curve (TAC) pattern of ACCs on 4-phase multidetector-row computed tomography (MDCT) were retrospectively reviewed, and the results were compared with those of ACs.

MATERIALS AND METHODS

Patients

The study design was approved by the institutional review board. Informed consent was not required because the review of the patients' data was anonymous. After a thorough search of the computerized database of the Hepatobiliary Pancreatic Surgery Division from April 2006 to March 2011, 6 patients with ACC and 88 patients with AC were identified on the basis of results obtained using surgically resected pancreatectomy specimens. Twenty-one AC patients were excluded because CT attenuation values for these tumors could not be measured accurately for the following reasons: halation of indwelling biliary drainage tube (11 ACs), small size and unclear tumor margin (9 ACs), and allergy to contrast media (1 AC). MDCT images of the 6 ACC patients and the remaining 67 AC patients were comparatively reviewed.

MDCT examination

All MDCT studies were performed using a scanner with 16 rows of detectors (Aquilion 16; Toshiba Medical Systems, Tokyo, Japan). CT images, both unenhanced and contrast enhanced, were routinely obtained with the patient in the supine position during full inspiration. For contrast-enhanced imaging, 100 mL of nonionic contrast material with iodine was administered at a rate of 3.2 cc/s using a mechanical power injector through a 20-gauge angiographic catheter inserted into a forearm vein. The scan delay time was 40 s for the arterial phase, 70 s for the portal venous phase, and 120 s for the delayed phase. Four-phase images (1 unenhanced image and 3 contrast-enhanced images) were routinely obtained. The scanning parameters for each phase were 1-mm collimation, 3-mm slice thickness, 3-mm reconstruction interval, 120 kV, and auto mA.

Imaging analysis

MDCT images were available from the picture archiving and communication system (PACS), and all images were reviewed on the PACS monitor. All CT images were evaluated retrospectively by 2 experienced hepatobiliary and pancreatic surgeons with 13 and 24 years of experience, respectively. CT images were assessed for the visual pattern and CT attenuation value of the ACs and ACCs. The visual pattern of each lesion was classified as hyperdense, isodense, or hypodense, compared to the surrounding normal pancreatic parenchyma in each phase. The CT attenuation value in Hounsfield units was obtained using region of interest (ROI) analysis. To reduce the effect of tumor heterogeneity, one ROI of the largest possible area was identified within the tumor at the level of maximum tumor diameter. The ROI value was calculated as the CT attenuation value of the tumor. Three ROIs of diameter 1 cm were also identified in the normal parenchyma adjacent to the tumor, and the mean of the 3 ROI values was calculated as the CT attenuation value of the surrounding parenchyma. While defining ROIs, special attention was paid to exclude cystic areas, calcification, the pancreatic duct, and the surrounding vessels. TAC patterns were drawn on the basis of each CT attenuation value, and they were compared between the ACCs and ACs.

Pathological examination and analysis

All the ACCs and ACs in this study were surgically resected, and 2 pathologists reviewed the gross appearance of the tumor specimens and hematoxylin-eosin-stained specimens on microscopic slides. For the ACCs, immunohistochemical analysis was performed for chromogranin and synaptophysin to exclude mixed acinar-endocrine carcinomas (MAEs).

Statistical analysis

The visual patterns of the ACCs and the ACs were compared using the χ^2 test. The CT attenuation values were

No.	Age, sex	Pre diag	Scheme	Location/size/surgery	Intra-tumoral necrosis	Intraductal tumor growth	Venous tumor thrombus
1	68, M	AC		Ph/35 mm/PD	○		
2	67, M	AC		Pb/48 mm/DP	○		○
3	77, M	AC		Pt/31 mm/DP	○		
4	61, M	AC		Phb/48 mm/PD		○	
5	52, M	AC		Pb/87 mm/DP	○	○	
6	89, M	ACC		Pb/32 mm/DP	○	○	

■ Primary tumor ▭ Intraductal tumor growth or venous tumor thrombus ○ Histologically proven tumor-related findings

Figure 1 Clinicopathological findings of the acinar cell carcinomas. M: Male; Pre Diag: Preoperative diagnosis; AC: Adenocarcinoma; ACC: Acinar cell carcinoma; Ph/b/t: Pancreatic head/body/tail; PD: Pancreaticoduodenectomy; DP: Distal pancreatectomy.

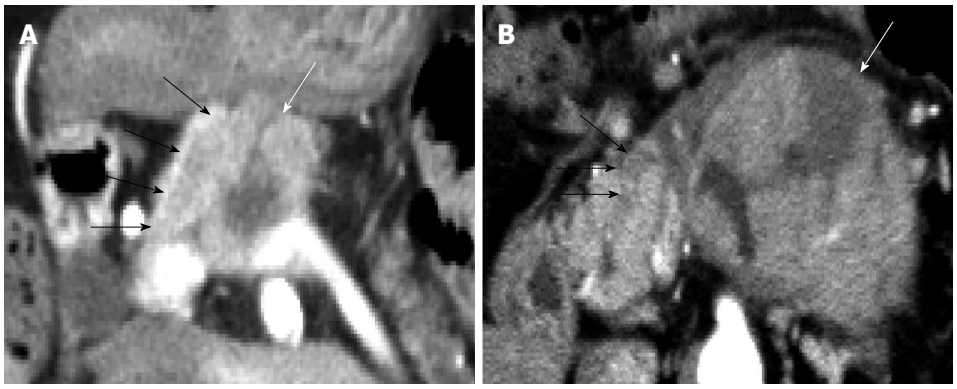

Figure 2 Acinar cell carcinomas with intraductal tumor growth. A: Case 6, computed tomography (CT) showed the primary acinar cell carcinoma (ACC) in the pancreatic body (white arrow) and the easily recognizable widespread intraductal tumor growth (ITG) (black arrows); B: Case 5, CT shows the primary ACC in the pancreatic body (white arrow) and the small almost-unrecognizable ITG (black arrows).

compared between each phase by using the Wilcoxon signed-rank test. The CT attenuation values of the ACCs and the ACs were compared by using the Mann-Whitney *U* test. Data were analyzed using IBM SPSS Statics 19, and *P* values less than 0.05 were considered statistically significant.

RESULTS

Clinicopathological findings

Each pancreatic tumor had been preoperatively diagnosed on the basis of blood examination, CT images and endoscopic findings at weekly hepatobiliary pancreatic conferences involving radiologists, gastroenterologists, and surgeons. Of the ACC cases, 5 tumors had been diagnosed as AC, and only 1 tumor (case 6) had been correctly diagnosed as ACC (Figure 1). In all 6 ACC cases, the patients were male (mean age, 69 years; range, 52-89 years). Two patients underwent pancreaticoduodenectomy, and the other 4 patients underwent distal pancreatectomy. The maximum diameter of the tumors ranged from 31 to 87 mm, and the mean maximum diameter was 46.8 mm. Five tumors showed intratumoral necrosis. Extraparenchymal tumor extension as ITG and VTT was observed in 3 patients and 1 patient, respectively (Figures 1 and 2). All ACCs were characterized by extensive cellularity and minimal stroma, and the tumor cells showed basophilic cytoplasm and frequently contained eosinophilic granules in the cytoplasm. The tumor cells were arranged in an acinar pattern in 3 ACCs and in a solid pattern in 3 ACCs. Immunohistochemical analysis showed negative reactions for chromogranin and synaptophysin in all cases. Re-examination of the morphological characteristics, cell structure, and immunohistochemical reactions of all resected

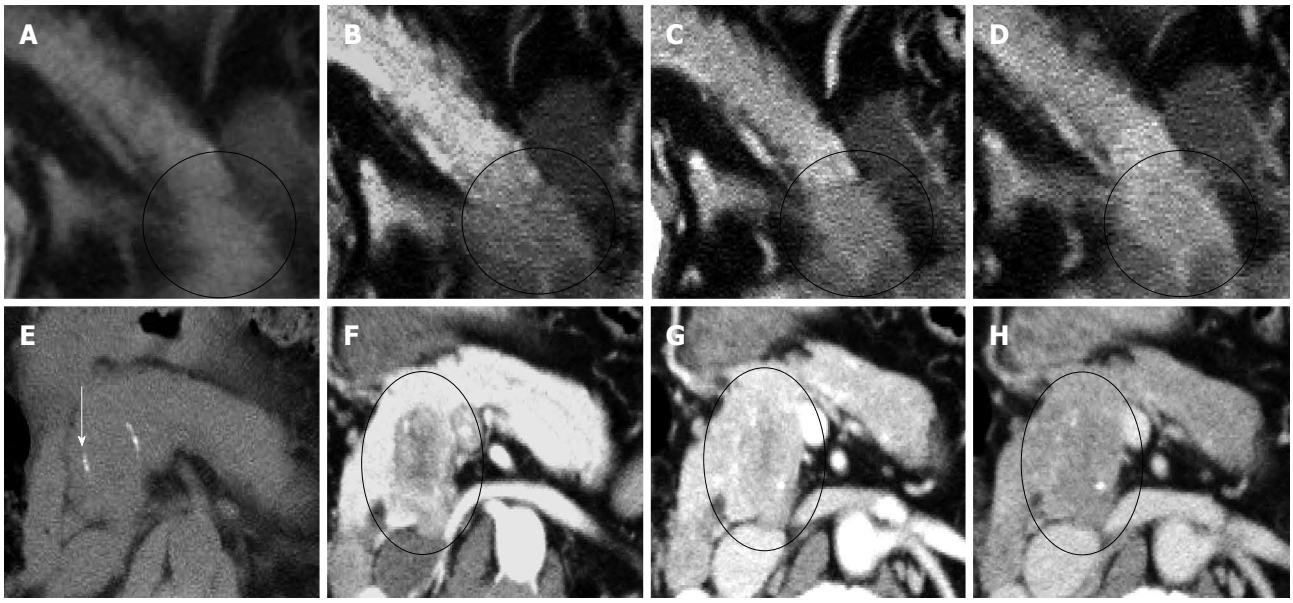

Figure 3 Visual patterns of the adenocarcinoma and the acinar cell carcinoma in the 4 phases. A-D: Adenocarcinoma in the pancreatic tail (circle); The tumor was hypodense in all 4 phases (A: Non-contrast phase; B: Arterial phase; C: Portal venous phase; D: delayed phase). It showed a gradual enhancement pattern across the phases; E-H: Case 1, Acinar cell carcinoma in the pancreatic head (circle); The tumor was isodense and undetectable in the non-contrast phase, although calcification was identified (arrow) (E); It was hypodense in all 3 contrast-enhanced phases (F: Arterial phase; G: Portal venous phase; H: Delayed phase); Contrast enhancement was the strongest in the portal venous phase (G).

Table 1 Visual pattern of acinar cell carcinoma and adenocarcinoma *n* (%)

	Non-contrast	Arterial	Portal venous	Delayed
ACC	Hypo	Hypo	Hypo	Hypo
	1 (17)	6 (100)	6 (100)	5 (83)
6 cases	Iso			Iso
	5 (83)			1 (17)
AC	Hypo	Hypo	Hypo	Hypo
	53 (79)	67 (100)	67 (100)	46 (69)
67 cases	Iso			Iso
	14 (21)			13 (19)
				Hyper
				8 (12)
P value	$P < 0.01$	NS	NS	NS

ACC: Acinar cellcarcinoma; AC: Adenocarcinoma; Hypo: Hypodense; Iso: Isodense; Hyper: Hyperdense; NS: Not significant.

tumors excluded neuroendocrine tumors and MAEs. All tumors were diagnosed as pure ACCs. Among 67 AC patients, 34 AC patients were male and 33 were female (mean age, 71.4 years; range, 34-87 years). The maximum diameter of the tumors ranged from 12 to 105 mm, and the mean maximum diameter was 35 mm. All tumors were whitish, solid, and associated with dense fibrotic stroma. None of the tumors was accompanied by significant intratumoral necrosis. All tumors were diagnosed as tubular adenocarcinoma; adenocarcinoma variants such as adenosquamous carcinoma, colloid carcinoma, and undifferentiated carcinoma were not observed.

MDCT findings

Visual pattern: Fifty-three ACs (79%) were hypodense while 14 (21%) were isodense in the non-contrast phase (Figure 3, Table 1). All ACs were hypodense in the arterial and portal venous phases. Forty-six ACs (69%), 13 ACs (19%), and 8 ACs (12%) were hypo-, iso-, and hyperdense in the delayed phase, respectively.

One ACC (17%) was hypodense and 5 (83%) were isodense in the non-contrast phase (Figure 3, Table 1). All ACCs were hypodense in all 3 contrast-enhanced phases, except for 1 tumor, which was isodense in the delayed phase. Thus, the visual pattern was clearly different between ACCs and ACs in the non-contrast phase ($P < 0.01$) (Table 1).

CT attenuation value and TAC pattern: The CT attenuation values of the ACs showed a gradually increasing pattern (non-contrast *vs* arterial, $P < 0.01$; arterial *vs* portal venous, $P < 0.01$; portal venous *vs* delayed, $P < 0.01$) (Figure 4). The TAC of all 67 ACs showed peak enhancement during the delayed phase.

The TAC of 4 ACCs showed peak enhancement during the portal venous phase. That of the remaining 2 ACCs showed peak enhancement during the arterial phase, followed by a gradual decline. Unlike the ACs, the ACCs showed significantly higher CT attenuation values in the portal venous phase than in the delayed phase ($P < 0.01$) (Figure 4).

In all 3 phases (non-contrast, arterial, and portal venous), the CT attenuation values of the ACCs were significantly higher than those of the ACs, although the visual patterns of the 2 tumors were clearly different only in the non-contrast phase (Figure 5, Table 1).

DISCUSSION

Previously, ACCs were considered equally aggressive

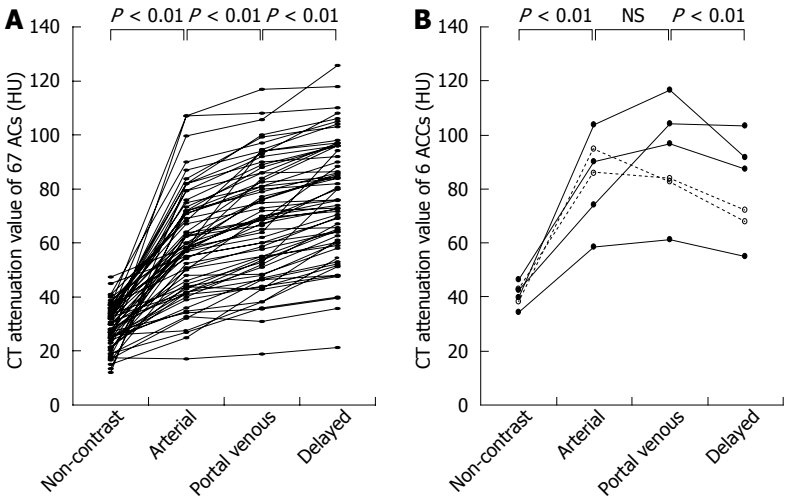

Figure 4 Time attenuation curve of the 67 adenocarcinomas (A) and 6 acinar cell carcinomas (B). Peak enhancement is seen during the delayed phase for all 67 acinar cell carcinomas. Meanwhile, peak enhancement is seen during the portal venous phase for 4 acinar cell carcinomas (ACCs) and during the arterial phase for 2 ACCs. None of the 6 ACCs show peak enhancement during the delayed phase. AC: Adenocarcinomas; CT: Computed tomography; NS: Not significant.

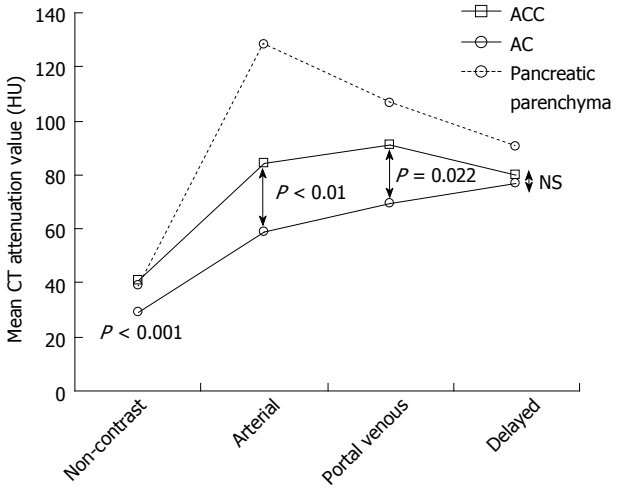

Figure 5 Mean computed tomography attenuation values of the tumors and the surrounding pancreatic parenchyma in the 4 phases. In 3 phases (non-contrast, arterial, and portal venous phase), the computed tomography (CT) attenuation values of the acinar cell carcinomas (ACCs) were significantly higher than those of the adenocarcinomas (ACs). NS: Not significant.

cancers as ACs[12,13]. Therefore, the treatment strategy for both tumors was essentially the same, and preoperative differentiation between ACC and AC was not considered important. However, in recent years, increasing evidence has shown that ACCs exhibit less aggressive growth and significantly better long-term survival than ACs[12]. Two recent large population-based studies proved the better prognosis of ACC[14,15]. Schmidt *et al*[14] reported the largest ACC series of 865 patients from the National Cancer Database, and they described the 5-year survival rates to be 36.2% and 10.4% for the resected and non-resected cases, respectively. The stage-specific 5-year survival was significantly better for resected ACC than AC (stage Ⅰ: 52.4% *vs* 28.4%; Ⅱ: 40.2% *vs* 9.8%; Ⅲ: 22.8% *vs* 6.8%; Ⅳ: 17.2% *vs* 2.8%). These findings suggest that the survival rate is better for ACC than for AC, and even in advanced ACC cases, survival can be improved by resection. Further, although no consensus has been reached on surgery for metastatic ACCs, a few reports have described a good prognosis after resection of limited metastatic disease. Hartwig *et al*[12] reported that the overall survival did not differ between 9 patients who underwent metastatic disease resection and 6 patients who underwent nonmetastatic disease resection. Suzuki *et al*[16] reported the case of a long-term survivor of metastatic ACC who was successfully treated with repetitive surgery. Because surgery might result in longer survival for ACC patients, even those with metastatic disease, the malignant potential of ACC and AC is thought to be significantly different, and accurate diagnosis of ACC is very important.

Recent reports on CT have shown that ACCs are typically solitary, and they are homogenously enhanced when the lesion is small but may contain hypodense areas because of necrosis if the lesion is large[11,17]. In terms of the visual pattern in contrast-enhanced phases, although a few reports described ACC to be a hyperdense tumor in the arterial phase[18], some reported that it tended to be enhanced less than the adjacent normal pancreatic parenchyma[10,11]. Chiou *et al*[17] reported on the CT manifestations of 8 ACCs, of which 6 were hypodense and 2 were isodense in the early arterial and portal venous phases. As shown in previous reports, ACCs tended to be hypodense in all 3 contrast-enhanced phases in the current study, and hypervascular pancreatic tumors, such as neuroendocrine tumor or metastatic renal cell carcinoma, were not included among the preoperative differential diagnoses. Although several such valid imaging findings of ACCs are available, accurate preoperative imaging-based diagnosis of ACCs, especially small ACCs, remains difficult[19]. In the current study, ACC was correctly diagnosed on the basis of recognizable widespread ITG on CT images in only 1 case (Figure 2A). Although the characteristic progression patterns of ITG or VTT were observed in 3 other cases (cases 2, 4, and 5), ACC was not preop-

eratively diagnosed in these cases. Because the ITG or VTT lesions were small and continuous with the primary tumor in these cases, they could not be considered to be the tumor that had progressed into the pancreatic duct or splenic vein, and they were regarded as part of the primary tumor (Figure 2B). To identify novel indicators for the accurate diagnosis of ACC, the CT attenuation values of ACC were compared with those of AC, which was the most frequently suspected disease in the preoperative diagnosis in our ACC cases, and we found that ACCs had a unique TAC pattern. The TAC of the ACCs showed the peak enhancement during the portal venous phase in the 4 ACCs, and during the arterial phase in the 2 ACCs. None of the 6 ACCs showed the peak enhancement during the delayed phase. This TAC pattern of ACC was clearly different from that of AC. Several studies have reported the CT findings of pancreatic AC, and it is well known that AC with fibrous stroma appears hypodense with delayed enhancement on dynamic CT[20-22]. The ACs in the current study also showed the gradual enhancement pattern, and all 67 ACs showed the peak enhancement during the delayed phase. Although the reasons for the different TAC pattern of ACs and ACCs have not been elucidated, we speculate that the degree of intratumoral fibrosis is one. Hattori *et al*[23] reported that the CT attenuation value of ACs correlated negatively with the extent of intratumoral fibrosis in 3 contrast-enhanced phases. The scanty fibrous stroma in the ACCs might have led to their higher CT attenuation values compared with those of the ACs. The isodensity of most ACCs in the non-contrast phase, which is clearly different from the hypodensity of most ACs, is also thought to reflect the degree of fibrosis. In this study, 3 relatively small ACCs (31, 32, and 35 mm in diameter) also showed the specific TAC pattern. Thus, this TAC pattern might be useful to distinguish ACCs from ACs, especially when they are small and have no distinguishing morphological features. Further, in the future, it may be possible to apply these different patterns of enhancement on MDCT to echoendoscopy.

Echoendoscopy has been reported to be superior to any other modality with respect to spatial resolution, and it can accurately detect small pancreatic lesions[24-26]. Contrast-enhanced endoscopic ultrasonography (CE-EUS) has emerged as a recent technological development, and this modality can be used to evaluate the degree of enhancement in pancreatic lesions[25,26]. Kitano *et al*[26] reported that CE-EUS was useful for characterizing pancreatic lesions and that it was superior to MDCT for diagnosing small lesions. Although, to our knowledge, no study has compared the enhancement pattern between ACs and ACCs using echoendoscopy, this modality may prove useful for distinguishing these 2 pancreatic tumors.

Despite the novel findings of this study, it does have some limitations. Firstly, the number of ACC cases included is small, and it is not clear whether or not every ACC definitely shows the unique TAC pattern. Another limitation is that the actual effectiveness of this TAC pattern is unclear, because of the retrospective nature of this study. Further investigation is necessary to prove that the TAC pattern is specific to ACCs and that it is actually useful in distinguishing ACCs from other pancreatic tumors.

In conclusion, the tumor density in the non-contrast phase and TAC pattern are clearly different between ACCs and ACs, although both tumors tend to be hypodense in the contrast-enhanced phases.

COMMENTS

Background

Acinar cell carcinoma (ACC) is a rare malignant epithelial neoplasm that exhibits exocrine enzyme production, and it accounts for approximately 1% of all pancreatic neoplasms. ACCs have been reported to be bulky tumors that mainly occur in the pancreatic head, and recent reports have shown that ACCs are often accompanied by intratumoral necrosis and have various specific extraparenchymal progression patterns, such as intraductal tumor growth and venous tumor thrombus. Several reports have described the computed tomography (CT) findings of ACC: it is typically solitary and is accompanied by an intratumoral hypodense area when large. In terms of the visual pattern, although a few hyperdense ACCs have been reported, most ACCs have been reported to be hypodense on contrast-enhanced CT.

Research frontiers

ACCs had been previously considered equally aggressive as ACs, and pretreatment differentiation between ACC and AC was not considered important. However, in recent years, increasing evidence has shown that ACCs are characterized by less aggressive growth and that ACC shows significantly better long-term survival than AC. Further, although no consensus has been reached on surgery for metastatic ACCs, a few reports have described a good prognosis after resection of limited metastatic disease. Because the malignant potential of ACC and AC is significantly different, correct pretreatment distinction between these two tumors is very important.

Innovations and breakthroughs

The tumor density in the non-contrast phase and time attenuation curve pattern clearly differ between acinar cell carcinomas and adenocarcinomas, and multidetector-row computed tomography can distinguish these tumors.

Applications

Each pancreatic tumor had been preoperatively diagnosed on the basis of blood examination, CT images and endoscopic findings at weekly hepatobiliary pancreatic conferences involving radiologists, gastroenterologists, and surgeons.

Peer review

This is an interesting paper. The number of acinar carcinomas is relatively small but still there are some interesting results. Suggest authors edit the paper before publication if they have more cases in hand.

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BRIEF ARTICLE

Laparoscopic-endoscopic cooperative surgery for gastric submucosal tumors

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Abstract

AIM: To assess the feasibility, safety, and advantages of minimally invasive laparoscopic-endoscopic cooperative surgery (LECS) for gastric submucosal tumors (SMT).

METHODS: We retrospectively analyzed 101 consecutive patients, who had undergone partial, proximal, or distal gastrectomy using LECS for gastric SMT at Peking Union Medical College Hospital from June 2006 to April 2013. All patients were followed up by visit or telephone. Clinical data, surgical approach, pathological features such as the size, location, and pathological type of each tumor; and follow-up results were analyzed. The feasibility, safety and effectiveness of LECS for gastric SMT were evaluated, especially for patients with tumors located near the cardia or pylorus.

RESULTS: The 101 patients included 43 (42.6%) men and 58 (57.4%) women, with mean age of 51.2 ± 13.1 years (range, 14-76 years). The most common symptom was belching. Almost all (n = 97) patients underwent surgery with preservation of the cardia and pylorus, with the other four patients undergoing proximal or distal gastrectomy. The mean distance from the lesion to the cardia or pylorus was 3.4 ± 1.3 cm, and the minimum distance from the tumor edge to the cardia was 1.5 cm. Tumor pathology included gastrointestinal stromal tumor in 78 patients, leiomyoma in 13, carcinoid tumors in three, ectopic pancreas in three, lipoma in two, glomus tumor in one, and inflammatory pseudotumor in one. Tumor size ranged from 1 to 8.2 cm, with 65 (64.4%) lesions < 2 cm, 32 (31.7%) > 2 cm, and four > 5 cm. Sixty-six lesions (65.3%) were located in the fundus, 21 (20.8%) in the body, 10 (9.9%) in the antrum, three (3.0%) in the cardia, and one (1.0%) in the pylorus. During a median follow-up of 28 mo (range, 1-69 mo), none of these patients experienced recurrence or metastasis. The three patients who underwent proximal gastrectomy experienced symptoms of regurgitation and belching.

CONCLUSION: Laparoscopic-endoscopic cooperative surgery is feasible and safe for patients with gastric submucosal tumor. Endoscopic intraoperative localization and support can help preserve the cardia and pylorus during surgery.

Key words: Laparoscopic-endoscopic cooperative surgery; Gastric submucosal tumor; Minimally invasive surgery; Laparoscopy; Endoscopy

Core tip: We retrospectively analyzed 101 consecutive patients who had undergone partial, proximal or distal gastrectomy using laparoscopic-endoscopic cooperative surgery (LECS) for gastric gastric submucosal tumor (SMT) at Peking Union Medical College Hospital from June 2006 to April 2013. Ninety-seven patients underwent surgery with preservation of the cardia and

pylorus, with the other four patients undergoing proximal or distal gastrectomy. LECS is feasible and safe for gastric SMT, especially for patients with tumors near the cardia or pylorus. Intraoperative localization and support by endoscopy can help preserve the cardia and pylorus during surgery.

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INTRODUCTION

Since the first gastrectomy was performed in 1880, surgical methods have developed rapidly due to improvement in anastomosis techniques, surgical staplers, and gastrointestinal tube application[1]. Moreover, since the first cholecystectomy by electronic laparoscopy was performed in 1987, minimally invasive laparoscopic surgery has become more popular for its lower postoperative morbidity rates and faster postoperative recovery[2-5]. Minimally invasive surgery is suitable for benign gastric lesions, especially for gastrointestinal stromal tumors (GISTs). Although GISTs are potentially malignant, nodal metastasis is rare. Therefore, excision of the tumor with negative margins but without lymphadenectomy has become a standard approach, while GISTs are indicated for minimally invasive partial gastrectomy[6-9].

Although gastric small mucosal tumors (SMT) have been resected laparoscopically, this type of surgery is associated with two potential problems. Laparoscopy may be unable to determine the location of gastric SMTs, because of their small size or intraluminal growth pattern. In addition, complications may arise during the laparoscopic removal of SMTs located near the cardia or pylorus; these complications can include stenosis or damage to the cardia or pylorus. We have therefore developed a technique, called minimally invasive laparoscopic-endoscopic cooperative surgery (LECS), for removal of SMTs. This paper represents our analysis of findings in 101 patients who successfully underwent LECS for gastric SMT at the Department of General Surgery, Peking Union Medical College Hospital, from June 2006 to April 2013.

MATERIALS AND METHODS

Clinical data

From June 2006 to April 2013, 101 patients successfully underwent LECS for gastric SMT at the Department of General Surgery, Peking Union Medical College Hospital; the cardia and pylorus were preserved in 97 of these patients. In addition to routine preoperative tests, all patients underwent upper gastrointestinal endoscopy with endoscopic ultrasound (EUS) and a computed tomography (CT) scan with three-dimensional gastric display. Demographic and clinicopathological characteristics were analyzed retrospectively. Demographic features assessed included patient sex and age, the length of the operation, estimated blood loss, and rate of conversion to open surgery. Postoperative data included time to bowel function recovery (normal passage of gas), surgical complications (*e.g.*, leakage, stenosis, and bleeding), and length of postoperative hospital stay. The clinicopathological characteristics of the SMTs included their size, location, and pathological type.

Surgical procedures

LECS was performed with the patient under general anesthesia in the reverse Trendelenburg position. The surgeon stood between the patient's legs, the first assistant was to the right or the left of the patient's body, the laparoscopist to the right of the patient's legs, and the gastroscopy to the left of the patient's head.

Setup for laparoscopic surgery

A camera port was inserted into the inferior (1 cm) umbilical incision (10 mm port) using an open technique. Three additional ports (two 5 mm and one 12 mm in diameter) were inserted into the left upper and right upper quadrants and the inferior xiphoid process (on the right or left side according to the location of the SMT), respectively, under a pneumoperitoneum of 1.60-1.86 kPa, with a laparoscopic view (30° angle range).

Endoscopic procedures

With the patient anesthetized, the endoscope was inserted through the oropharynx. The mucosae of the esophagus and stomach were viewed, taking care not to infuse too much air into the stomach. The location of the SMT was confirmed, all liquids and gas were withdrawn, and the endoscope was withdrawn through the cardia to remain in the esophagus[10].

Operative approaches

Tumors within the anterior wall of the stomach: The omentum was detached and a little air was allowed to fill the stomach endoscopically. Using both the laparoscope and the endoscope, the location of the SMT was confirmed by the method of touch and marked by one or two suture lines. The gastric wall, including the SMT, was elevated with two seromuscular sutures placed opposite each other and 2-4 cm from the lesion. The tumors, as well as some normal gastric tissues, were removed with a linear endoscopic gastrointestinal stapler (*e.g.*, EC_{60}). If the tumor was located near the esophagogastric junction or pyloric ring, the endoscope was placed distally into the stomach or duodenum to protect the normal gastric tissues from stenosis or damage. After the lesion was resected, direct intraluminal visualization was performed to ensure that the tumor was totally removed and that there was no bleeding or leakage. The amount of air in

Table 1 Demographic and clinical characteristics of the 101 patients who underwent laparoscopic and endoscopic cooperative surgery for gastric submucosal tumors *n* (%)

Parameters	Statistics
No. of patients	101
Age (yr)	51.2 ± 13.1 (range 14-76)
Sex	
Male	43 (42.6)
Female	58 (57.4)
Chief complaint	
Dyspepsia (regurgitation, eructation, belching, epigastralgia, and epigastric discomfort)	69 (68.3)
Physical examination (asymptomatic)	27 (26.7)
Melena	5 (5.0)
Tumor location	
Cardia	3 (3.0)
Gastric fundus	66 (65.3)
Gastric body	21 (20.8)
Gastric antrum	10 (9.9)
Pylorus	1 (1.0)
Distance between the tumor and cardia or pylorus (cm)	3.4 ± 1.3 (minimum 1.5)

Data are presented as mean ± SD.

the stomach and peritoneum was balanced, resulting in a good visual field.

Tumors within the posterior wall of the stomach: The proximate curvature was detached to expose the tumor, using, for example, the Ligasure vascular sealing system. The posterior wall was rotated, and the tumor was resected using a technique similar to that described for anterior lesions.

Tumors within the lesser curvature (anterior and posterior gastric wall borderline) of the stomach: The small omentum was detached to expose the tumor, followed by tumor resection using the technique described above. For larger tumors, the left gastric vessels were cut off to prevent both operative and postoperative bleeding. Endoscopic support was especially important for tumors located near the esophagogastric junction[11].

The resected tumor was placed in a specimen retrieval bag located outside the left upper quadrant port. The tumor was cut open along the suture lines, and any ruptures in tumor integrity were assessed. The tumor was measured, immersed in 10% formalin solution, and sectioned. The sections were routinely stained with hematoxylin and eosin, and the number of mitotic figures per 50 high powered fields (HPF) was counted. Risk classifications for GIST were those described by the National Institutes of Health (NIH) in 2008. Gastric GIST was confirmed by immunohistochemistry, using antibodies to identify CD-117 (c-kit), CD-34, and DOG-1.

Follow-up

All patients were followed up by visit or telephone after 1, 3, 6, 9, 12, 24, 36, 48, and 60 mo. Each follow-up included a medical history review of any reports of abdominal discomfort, as well as CT scans and upper gastrointestinal endoscopy to exclude tumor recurrence or metastasis.

Statistical analysis

Data are expressed as mean ± SD. All analyses were performed using SPSS 12.0 software (SPSS, Chicago, IL, United States).

RESULTS

Surgery was successful in all 101 patients. The demographic and clinical characteristics of the 101 patients are depicted in Table 1. Three patients each had two GISTs.

Of the 101 patients, four underwent proximal or distal gastrectomy, including three with tumors located at the cardia, and one with a tumor located at the pylorus. The remaining 97 patients had preservation of the cardia and pylorus. During surgery, tumor location could not be confirmed by laparoscopy alone in 92 patients.

The mean operation time was 113 ± 36 min, and none of these patients required conversion to open surgery. Mean estimated blood loss was 36 ± 18 mL. The postoperative course of all patients was uneventful, with no anastomosis leakage. One patient who underwent proximal gastrectomy had an anastomotic stenosis because of scar physique. This patient was successfully treated by balloon dilatation under X-ray fluoroscopy. One patient experienced anastomotic bleeding and was successfully treated by conservative methods (drug hemostasis and blood transfusion). The average time to first gas passage was 2.9 ± 0.9 d, the average time for nasal-gastric tube placement was 1.9 ± 0.5 d, and the average postoperative hospital stay was 4.2 ± 1.1 d (Table 2). Seven patients underwent simultaneous laparoscopic cholecystectomy for gallstones, and two underwent simultaneous endoscopic polypus dissection.

All the resected tumors were cut open along the suture lines, with none showing evidence of rupture.

The clinicopathological characteristics of the submucosal stomach tumors, including their location, are shown in Table 3. Of the 101 tumors, 78 (77.2%) were GISTs, with 53 located in the gastric fundus, 14 in the gastric body, seven in the antrum, three in the cardia, and one in the pylorus. The remaining tumors included 13 (12.9%) leiomyomas, 11 in the gastric fundus and two in the gastric body; three (3.0%) ectopic pancreases, two in the gastric fundus and one in the antrum; three (3.0%) carcinoids, two in the gastric body and one in the antrum; two (2.0%) lipomas, one each in the gastric body and antrum; one (1.0%) glomus tumor in the gastric body; and one (1.0%) inflammatory pseudotumor in the gastric body. Maximum tumor size ranged from 1 to 8.2 cm, with 65 (64.4%) lesions < 2 cm in size, 32 (31.7%) > 2 cm, and four > 5 cm.

Gastric GIST was confirmed by immunohistochemistry in 78 patients, with 68 (87.2%) positive for CD117, 65 (82.9%) positive for CD34, and 65 (82.9%) positive for DOG1. Using the NIH biological risk classification for GIST[12], we found that 54 (69.2%) tumors were of

Table 2 Operative data for laparoscopic and endoscopic cooperative surgery *n* (%)

Parameters	Statistics
Operation time (min)	113 ± 36
Conversion to open surgery	0 (0)
Intraoperative blood loss (mL)	36 ± 18
Postoperative complications	
Gastric fullness	0 (0)
Anastomotic leakage	0 (0)
Anastomotic stenosis	1 (1.0)
Anastomotic bleeding	1 (1.0)
Postoperative hospital stay (d)	4.5 ± 2.1
Time for nasal-gastric tube placement (d)	1.9 ± 0.5
Time until bowel function recovery (d)	2.9 ± 0.9

Data are presented as mean ± SD.

Table 3 Clinicopathologic characteristics of submucosal tumors *n* (%)

Parameters	Statistics
Pathological diagnosis	
Gastrointestinal stromal tumor	78 (77.1)
Leiomyoma	13 (12.9)
Ectopic pancreas	3 (3.0)
Carcinoid	3 (3.0)
Lipoma	2 (2.0)
Glomus tumor	1 (1.0)
Inflammatory pseudotumor	1 (1.0)
Tumor size (cm)	4.9 ± 0.6

Data are presented as mean ± SD.

very low risk, including 41 in the gastric fundus, seven in the gastric body, four in the antrum, and two in the cardia; and 16 (23.5%) were of low risk, including eight in the gastric fundus, four in the gastric body, two in the antrum, one in the cardia, and one in the pylorus. Six tumors (7.7%), of mean size 5.4 ± 1.3 cm, were of moderate risk, including three in the gastric fundus, two in the gastric body, and one in the antrum. Two tumors (2.6%) were of high risk, one located in the gastric fundus was 8.2 cm in size; and the second, located in the gastric body, showed 13 mitotic figures/50 HPF. The first patient was treated with imatinib for 2 mo before the surgery, which decreased the tumor size from 8.8 to 8.2 cm in diameter. The eight patients in the moderate- and high-risk classes were treated with adjuvant imatinib for 1-2 years.

All the patients were followed up after LECS, for a mean time of 28 mo (range, 1-69 mo). The three patients who underwent proximal gastrectomy developed symptoms of regurgitation, eructation, and belching. None of the 101 patients who underwent LECS showed evidence of tumor recurrence, metastasis, nutritional disturbances (*e.g.*, weight loss, vitamin deficiency, deficiency of trace elements), or decreased quality of life. One patient developed primary liver cancer 2 years and 4 mo after LECS, but this patient remains alive. In addition, none of the patients with preserved cardia and pylorus experienced any symptoms of epigastric discomfort.

DISCUSSION

We have shown here that LECS is feasible, yielding satisfactory surgical results, in patients with gastric SMT. Usually, gastric SMTs are resected by open surgery, either distal or proximal gastrectomy[13]. Operation time and postoperative hospital stay are longer, and many patients develop gastroesophageal reflux disease (GERD). Quality of life may decrease, and the risk of remnant gastric cancer or esophageal carcinoma may increase. In contrast, LECS requires a relatively small resection of the healthy gastric wall, with very low rates of postoperative morbidity and mortality. Of our 101 patients, only two experienced postoperative complications, one with anastomotic stenosis and one with anastomotic bleeding. Although tumors with an extragastric growth pattern can be easily treated using conventional laparoscopic wedge resection, laparoscopic methods alone have some limitations for the resection of gastric SMTs. Laparoscopy has been found to be less efficient than open surgery in removing small tumors and tumors located in the posterior gastric wall and lesser curvature of the stomach. In addition, the removal of large tumors and those located near the cardia or pylorus can result in post-operative complications, such as stenosis or damage to the cardia or pylorus.

All of our patients routinely underwent two important preoperative tests, upper gastrointestinal endoscopy with EUS and CT scan with a three-dimensional gastric display, both of which are very important for this surgery. EUS was used to assess depth of tumor invasion, lesion location, tumor size, and growth pattern[14-18]. The diagnostic accuracy of EUS, however, may be affected by technical problems or skills or the subjective view of the operator, whereas the diagnostic accuracy of CT scanning was less subjective. CT three-dimensional imaging was helpful in assessing tumor size, the distance between the tumor and local tissues (cardia and pylorus), and the diagnosis and staging of SMTs. Use of these two tests could therefore determine whether localized gastric SMTs can be resected.

Endoscopic submucosal dissection (ESD) performed by experienced endoscopists has been used to remove gastric SMTs[19-22]. We found that 78 of our 101 (77.2%) SMTs were GISTs. GISTs are a type of mesenchymal neoplasm, originating from Cajal cells; are located in the submucous, muscularis propria, or subserous layer; and have an intraluminal or extrinsic growth pattern. ESD resection of tumors in the muscularis propria, while preserving the integrity of the serous layer, is very difficult. ESD alone may result in high rates of resection failure, intraoperative bleeding, and perforation. In addition, this procedure cannot easily differentiate between benign and malignant tumors. Since GISTs are regarded as potentially malignant and in need of complete resection, ESD alone should not be used to remove gastric SMTs.

The development of the LECS procedure has expanded the range of minimally invasive surgery. The endoscopic assistant cut the exact edges from the gastric

lumen, followed by tumor resection aided by endoscopy. Endoscopic support could reduce complications, such as stenosis or damage to the cardia or pylorus, especially when the tumor is located in the gastric fundus or antrum. Moreover, direct intraluminal visualization can confirm that the tumor has been totally removed, that there is no bleeding from the suture lines, and that there are no perforations. When observing through the endoscope, the pneumoperitoneum should be at lower pressure and the laparoscope should be removed for a better view. All gas and liquid should be removed endoscopically for better laparoscopic procedures. Laparoscopy may be sufficient, however, for large tumors, for tumors located near the cardia and pylorus, and for tumors with an extrinsic growth pattern. Even in these situations, however, endoscopic support is important for protecting the cardia and/or pylorus from damage during resection, even if the endoscope is not need to confirm tumor location. LECS can therefore improve the success rates and outcomes of minimally invasive surgery without postoperative morbidity or mortality.

The sphincter muscles in the cardia and pylorus are important anatomical structures for preserving regurgitation. Although 59.1% of SMTs were reported located at the fundus[11], we found that the percentage was higher, 67.9%. Resection of the cardia can cause symptoms like heartburn due to gastric acid regurgitation. These patients may have to take medicines like proton pump inhibitors for a long time, reducing patient quality of life, and may develop GERD or esophageal carcinoma. Of our 101 patients, only three underwent proximal gastrectomy, with all three developing symptoms of regurgitation, eructation, and belching. Similar findings would be observed after resection of the pylorus, since duodenal juice would regurgitate into the remnant stomach, causing inflammation at the suture lines and corresponding symptoms and ultimately leading to remnant gastric cancer[21,22]. Therefore, it is very important to preserve these important anatomical structures. LECS can decrease the risk to resect the cardia and pylorus. We found that the minimum distance from the edge of the tumor to the cardia was 1.5 cm. The importance of endoscopic support was inversely correlated with the distance between the tumor edge and the cardia or pylorus[23]. In addition, GISTs are supplied by many blood vessels. When resecting larger tumors within the lesser curvature, the left gastric vessels should be cut off to prevent postoperative bleeding. In this study, one 76-year-old patient experienced anastomotic bleeding, because of atherosclerosis. After 2 d of conservative therapy, consisting of blood transfusions, he got better and was discharged.

All 101 of our patients underwent minimally invasive surgery, with LECS in 97 resulting in the preservation of the cardia and pylorus. None of these patients required conversion to open surgery. Intraoperative bleeding was limited and recovery of bowel function was rapid, with a low postoperative morbidity (except for one patient each with anastomotic stenosis and bleeding), and no postoperative mortality. Postoperative hospital stay was much shorter than in several previous studies. Except for the three patients who underwent proximal gastrectomy, none developed symptoms like GERD and their quality of life did not decrease over a relatively long-term follow-up, suggesting the importance of preserving the anatomical structure and physical function of the cardia and pylorus. None of our 78 patients with gastric GIST developed tumor recurrence or metastasis after LECS, regardless of risk classification, indicating that total resection of SMTs, including potentially malignant GISTs, by the LECS techniques yields satisfactory surgical outcomes. We found that 50% of tumors classified as moderate or high risk, and most with more than five mitoses per 50 HPFs, were located at the gastric fundus. Patients in moderate- and high-risk categories required adjuvant imatinib[24]. We found that two patients had tumors < 5 cm, but more than 10 mitotic figures per 50 HPFs.

LECS can be used for two types of partial gastrectomy. The first consists of laparoscopic wedge resection of gastric SMTs and distal or proximal gastrectomy under endoscopic guidance; and the second consists of laparoscopic cutting of the anterior wall of the stomach, to expose SMTs in the posterior gastric wall, followed by partial resection of the posterior gastric wall. All 101 of our patients with SMTs underwent complete resection, even if the tumors were located in the posterior, the lesser curvature of the stomach or near the cardia or pylorus. The greater curvature of the stomach was detached, the stomach was turned axially, and wedge resection was performed. A good view during this procedure requires that the amount of air in the stomach and peritoneum should be balanced.

LECS is indicated for the removal of SMTs (*e.g.*, leiomyomas, lipomas, and schwannomas), polyps with broad stalks, gastric epithelial tumor degeneration (moderate or severe atypical hyperplasia), lesions with low potential for malignancy (*e.g.*, carcinoid tumors and GISTs), and early-stage, localized gastric carcinomas[25]. Because GISTs may easily rupture during laparoscopic surgery, resulting in peritoneal seeding, the integrity of a resected GIST is regarded as a significant prognostic factor. Before 2007, the guidelines of the National Comprehensive Cancer Network did not recommend laparoscopic surgery for GIST resection, except for tumors < 2 cm in diameter and with a low risk of rupture. Although almost one-third of the tumors in this study were > 2 cm in diameter, LECS was successful for all tumors, regardless of tumor size. These findings indicate that the performance of laparoscopic and endoscopic techniques by skilled operators, non-contact with the tumor during surgery, and the use of a specimen retrieval bag are key factors for good surgical results. Tumors > 5 cm in diameter require resection of a relatively large portion of healthy stomach to ensure tumor integrity without rupture[26].

This study had several limitations, including its retrospective design and lack of comparisons with open or laparoscopic surgery. Prospective, multicenter, compara-

tive studies are needed to evaluate the role of LECS for gastric SMT.

In conclusion, we have shown here that LECS is a safe, easy, and beneficial procedure for gastric SMTs. Endoscopy functions to locate the tumor and to support the gastric lumen. The LECS technique, therefore, provides an alternative gastric wedge resection procedure with minimal transformation of the stomach.

COMMENTS

Background

Although gastric small mucosal tumors (SMT) have been resected laparoscopically, this type of surgery is associated with two potential problems. Laparoscopy may be unable to determine the location of gastric SMTs, because of their small size or intraluminal growth pattern. In addition, complications may arise during the laparoscopic removal of SMTs located near the cardia or pylorus; these complications can include stenosis or damage to the cardia or pylorus.

Research frontiers

Laparoscopic-endoscopic cooperative surgery (LECS) is indicated for the removal of SMTs (*e.g.*, leiomyomas, lipomas, and schwannomas), polyps with broad stalks, gastric epithelial tumor degeneration (moderate or severe atypical hyperplasia), lesions with low potential for malignancy (*e.g.*, carcinoid tumors and gastrointestinal stromal tumors), and early-stage, localized gastric carcinomas.

Innovations and breakthroughs

The authors have developed a technique, called minimally invasive LECS, for removal of SMTs. This paper represents the analysis of findings in 101 patients who successfully underwent LECS for gastric SMT at the Department of General Surgery, Peking Union Medical College Hospital, from June 2006 to April 2013.

Applications

The study results suggest that the LECS is feasible and safe for patients with gastric SMT. Endoscopic intraoperative localization and support can help preserve the cardia and pylorus during surgery.

Terminology

LECS represents a technique, called minimally invasive laparoscopic-endoscopic cooperative surgery for removal of gastric small mucosal tumors.

Peer review

This is a very interesting paper about laparoscopic-endoscopic surgery for gastric submucosal tumor. In this manuscript, the authors analyzed 101 patients who underwent laparoscopic-endoscopic surgery for gastric submucosal tumor. The authors discussed the safety and advantages of minimally invasive laparoscopic-endoscopic cooperative surgery for gastric submucosal tumor. The clinical data was well collected, and the surgical procedures of the patients were well described. The references are updating.

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BRIEF ARTICLE

Clonal immunoglobulin heavy chain and T-cell receptor γ gene rearrangements in primary gastric lymphoma

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Abstract

AIM: To study the diagnostic value of immunoglobulin heavy chain (IgH) and T-cell receptor γ (*TCR-γ*) gene monoclonal rearrangements in primary gastric lymphoma (PGL).

METHODS: A total of 48 patients with suspected PGL at our hospital were prospectively enrolled in this study from January 2009 to December 2011. The patients were divided into three groups (a PGL group, a gastric linitis plastica group, and a benign gastric ulcer group) based on the pathological results (gastric mucosal specimens obtained by endoscopy or surgery) and follow-up. Endoscopic ultrasonography (EUS) and EUS-guided biopsy were performed in all the patients. The tissue specimens were used for histopathological examination and for *IgH* and *TCR-γ* gene rearrangement polymerase chain reaction analyses.

RESULTS: EUS and EUS-guided biopsy were successfully performed in all 48 patients. In the PGL group (n = 21), monoclonal IgH gene rearrangements were detected in 14 (66.7%) patients. A positive result for each set of primers was found in 12 (57.1%), 8 (38.1%), and 4 (19.0%) cases using FR1/JH, FR2/JH, and FR3/JH primers, respectively. Overall, 12 (75%) patients with mucosal-associated lymphoid tissue lymphoma (n = 16) and 2 (40%) patients with diffuse large B-cell lymphoma (n = 5) were positive for monoclonal IgH gene rearrangements. No patients in the gastric linitis plastica group (n = 17) and only one (10%) patient in the benign gastric ulcer group (n = 10) were positive for a monoclonal *IgH* gene rearrangement. No *TCR-γ* gene monoclonal rearrangements were detected. The sensitivity of monoclonal IgH gene rearrangements was 66.7% for a PGL diagnosis, and the specificity was 96.4%. In the PGL group, 8 (100%) patients with stage IIE PGL (n = 8) and 6 (46.1%) patients with stage IE PGL (n = 13) were positive for monoclonal *IgH* gene rearrangements.

CONCLUSION: *IgH* gene rearrangements may be associated with PGL staging and may be useful for the diagnosis of PGL and for differentiating between PGL and gastric linitis plastica.

Key words: Immunoglobulin heavy chain; T-cell receptor γ; Gene rearrangement; Primary gastric lymphoma; Endoscopic biopsy specimen

Core tip: In 2003, a new primer system was successfully developed and standardized for the detection of clonally rearranged immunoglobulin (Ig) and T-cell receptor (*TCR*) genes. This study was a prospective analysis of Ig heavy chain (*IgH*) and *TCR-γ* gene rearrangements using the new primer system and endoscopic biopsy specimens from patients with suspected primary gastric lymphoma (PGL). Our study revealed that the detec-

tion of monoclonal *IgH* gene rearrangements is useful for the diagnosis of PGL and for differentiating between PGL and gastric linitis plastica. Monoclonal *IgH* gene rearrangements may be associated with PGL staging. The sensitivity and the specificity of *IgH* gene rearrangements for the diagnosis of PGL were 66.7% and 96.4%, respectively.

Shan GD, Hu FL, Yang M, Chen HT, Chen WG, Wang YG, Chen LH, Li YM, Xu GQ. Clonal immunoglobulin heavy chain and T-cell receptor γ gene rearrangements in primary gastric lymphoma. *World J Gastroenterol* 2013; 19(34): 5727-5731 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i34/5727.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i34.5727

INTRODUCTION

Primary gastric lymphoma (PGL) is a relatively rare tumor type that accounts for 5% of all gastric tumors[1]. Gastroscopy and biopsy are the primary methods for diagnosis. However, most PGLs arise in the submucosa, and the diagnosis of PGL by gastroscopy and biopsy is often difficult. An endoscopic presentation of polypoid lesions, flat lesions, enlarged gastric folds, ulcers, erosions, and negative or inconclusive histology may lead clinicians to suspect gastric lymphoma[2-8]. It is recommended that biopsy specimens undergo histomorphological, immunohistochemical, and immunophenotypic analyses for a diagnosis of gastric lymphoma. However, these methods may not lead to a diagnosis, especially in the early stages of the disease.

Immunoglobulin (Ig) and T cell receptors (TCRs) are the molecules responsible for B- and T-cell immune responses. The analysis of antigen receptor gene rearrangements by polymerase chain reaction (PCR) is a routine diagnostic tool for lymphoproliferative disorders[9].

Previous studies have demonstrated that Ig gene rearrangements in endoscopic biopsy samples were an additional tool for the diagnosis of gastric mucosal-associated lymphoid tissue (MALT) lymphoma[10-15]. In previous studies, the primers and PCR conditions were not standardized. Multiplex PCR assays have been available since 2003, and these assays have been standardized for the detection of clonally rearranged *Ig* and *TCR* genes[16]. However, multiplex PCR assays for the detection of Ig heavy chain (*IgH*) and *TCR-γ* gene rearrangements in PGL have not been previously reported. The aim of this study was to investigate the detection rate and the diagnostic value of *IgH* and *TCR-γ* gene monoclonal rearrangements in PGL endoscopic biopsy specimens.

MATERIALS AND METHODS

Patients

A total of 48 patients with suspected PGL at our hospital were prospectively enrolled in this study from January 2009 to December 2011. The patients were divided into three groups based on the pathological results (gastric mucosal specimens obtained by endoscopy or surgery) and follow-up. The PGL group consisted of 21 patients (14 males, 7 females, a mean age of 51 years, range 20-81 years). The gastric linitis plastica group consisted of 17 patients (11 males, 6 females, a mean age of 53 years, range 17-79 years). The benign gastric ulcer group consisted of 10 patients (7 males, 3 females, a mean age of 47 years, range 22-76 years).

Methods

Patients who met the criteria for suspected gastric lymphoma (an endoscopic presentation of polypoid lesions, flat lesions, enlarged gastric folds, ulcers, erosions, and negative or inconclusive histology) were included in the study. Patients with palpable superficial lymphadenopathy, obvious mediastinal lymphadenopathy, abnormal total and differential white blood cell counts, and the involvement of other organs in the abdomen were excluded from the study.

Informed consent was obtained from all the patients and this study was approved by the hospital before endoscopic ultrasonography (EUS) and the medical records analysis. EUS and EUS-guided biopsy were performed in all the patients. Overall, 8-10 biopsies were obtained from each patient. The specimens were submitted for histopathological examination. A portion of each specimen was stored at -80 ℃ for the gene rearrangement analysis by PCR.

DNA was isolated from frozen tissue by cell lysis, phenol extraction, and ethanol precipitation according to standard procedures. Alternatively, reactive DNAzol (Songon Biotech, Shanghai, China) was used according to the manufacturer's specifications. In each experiment, polyclonal DNA (reactive lymphoid tissue) and negative (sterile water) and positive controls were systematically included. To analyze the *IgH* gene, three sets of VH primers and one JH consensus primer were combined in three multiplex tubes. To analyze the *TCR-γ* gene, four Vγ primers and two Jγ primers were divided into two tubes (Table 1).

The PCR conditions were × 1 PCR buffer [50 mmol/L KCl, 10 mmol/L Tris (pH, 8.3), 1.5 mmol/L $MgCl_2$], 200 μmol/L of each deoxynucleotide, 10 pmol of each primer, and 2 U of Taq polymerase (Songon Biotech, Shanghai, China). The total PCR reaction volume was 50 μL. The thermal cycling conditions were pre-activation for 7 min at 95 ℃, followed by annealing at 60 ℃. Each reaction consisted of 35 cycles. The cycles were preceded by an initial denaturation step for 45 s, followed by a terminal extension for 10 min.

Patients with PGL were staged at baseline using computed tomography scans of the neck, the thorax, and the abdomen, followed by EUS and bone marrow biopsy. EUS staging was performed according to the Ann Arbor staging system[10].

RESULTS

EUS and EUS-guided biopsy were successfully performed in all 48 patients. In the PGL group, monoclonal

Table 1 Primer sequences

Gene	Sequence
IgH tube A	
VH1-FR1	5′GGCCTCAGTGAAGGTCTCCTGCAAG3′
VH2-FR1	5′GTCTGGTCCTACGCTGGTGAAACCC3′
VH3-FR1	5′CTGGGGGGTCCCTGAGACTCTCCTG3′
VH4-FR1	5′CTTCGGAGACCCTGCCCTCACCTG3′
VH5-FR1	5′CGGGGAGTCTCTGAAGATCTCCTGT3′
VH6-FR1	5′TCGCAGACCCTCTCACTCACCTGTG3′
JH consensus	5'CTTACCTGAGGAGACGGTGACC3'
IgH tube B	
VH1-FR2	5′CTGGG TGCGA CAGGC CCCTG GACAA3′
VH2-FR2	5′TGGAT CCGTC AGCCC CCAGG GAAGG3′
VH3-FR2	5′GGTCC GCCAG GCTCC AGGGA A3′
VH4-FR2	5′TGGAT CCGCC AGCCC CCAGG GAAGG3′
VH5-FR2	5′GGGTG CGCCA GATGC CCGGG AAAGG3′
VH6-FR2	5′TGGAT CAGGC AGTCC CCATC GAGAG3′
VH7-FR2	5′TTGGG TGCGA CAGGC CCCTG GACAA3′
JH consensus	5'CTTACCTGAGGAGACGGTGACC3'
IgH tube C	
VH1-FR3	5′TGGAG CTGAG CAGCC TGAGA TCTGA3′
VH2-FR3	5′CAATG ACCAA CATGG ACCCT GTGGA3′
VH3-FR3	5′TCTGC AAATG AACAG CCTGA GAGCC3′
VH4-FR3	5′GAGCT CTGTG ACCGC CGCGG ACACG3′
VH5-FR3	5′CAGCA CCGCC TACCT GCAGT GGAGC3′
VH6-FR3	5′GTTCT CCCTG CAGCT GAACT GTGTG3′
VH7-FR3	5′CAGCA CGGCA TATCT GCAGA TCAG3′
JH consensus	5'CTTACCTGAGGAGACGGTGACC3'
TCR-γ tube A	
Vγ1f	5′GGAAG GCCCC ACAGC RTCTT3′
vγ10	5′AGCATGGGTAAGACAAGCAA3′
Jγ1.1/2.1	5′TTACCAGGCGAAGTTACTATGAGC3′
Jγ:1.3/2.3	5′GTGTTGTTCCACTGCCAAAGAG3′
TCR-γ tube B	
Vγ9	5′CGGCA CTGTC AGAAA GGAATC3′
Vγ11	5′CTTCC ACTTC CACTT TGAAA3′
Jγ1.1/2.1	5′TTACCAGGCGAAGTTACTATGAGC3′
Jγ:1.3/2.3	5′GTGTTGTTCCACTGCCAAAGAG3′

The detection of immunoglobulin heavy chain (*IgH*) gene rearrangement using three sets of VH primers and one JH consensus primer combined in three multiplex tubes. The detection of T-cell receptor γ (*TCR-γ*) gene rearrangement using four Vγ primers and two Jγ primers, which were divided into two tubes.

IgH gene rearrangements were detected in 14 (66.7%) patients. Positive results for each set of primers were obtained in 12 (57.1%), 8 (38.1%), and 4 (19.0%) cases using FR1/JH, FR2/JH, and FR3/JH primers, respectively (Figure 1). No patients in the gastric linitis plastica group were positive for a monoclonal *IgH* gene rearrangement. In the benign gastric ulcer group, a monoclonal *IgH* gene rearrangement was detected in one (10%) patient. Overall, no *TCR-γ* gene monoclonal rearrangements were detected. For the diagnosis of primary gastric lymphomas, the sensitivity of the monoclonal *IgH* gene rearrangements was 66.7% and the specificity was 96.4%.

In the PGL group, the clinical stage distribution was as follows: IE in 13 patients and IIE in 8 patients. All 8 (100%) patients with stage IIE PGL were positive for monoclonal *IgH* gene rearrangements. Additionally, 6 (46.1%) patients with stage IE PGL were positive for monoclonal *IgH* gene rearrangements. The PGL group consisted of 16 patients with MALT lymphoma and 5 patients with diffuse large B-cell lymphoma (DLBCL). In the patients with MALT lymphomas, 12 (75%) were positive for monoclonal *IgH* gene rearrangements. In the patients with DLBCL, 2 (40%) were positive for monoclonal *IgH* gene rearrangements.

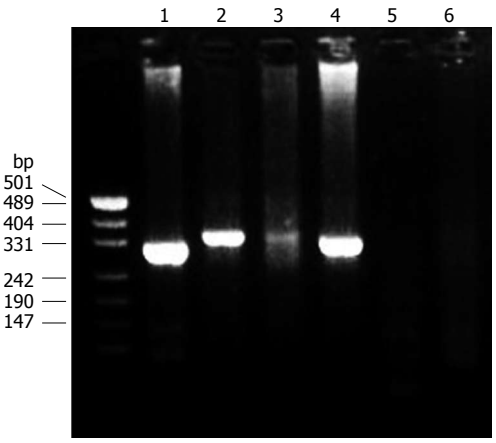

Figure 1 The results of immunoglobulin heavy chain gene rearrangement using FR1/JH primers. Lanes 1, 5, and 6 indicate a positive B-cell gastric lymphoma cell line, a negative control (sterile water), and a polyclonal control, respectively. Lanes 2, 3, and 4 show the presence of a monoclonal rearranged band that is within the expected range of size (242-331 bp).

In the benign gastric ulcer group, patients were treated with a proton-pump inhibitor (esomeprazole 20 mg daily) for 8-16 wk. Repeated endoscopies revealed that the gastric ulcers were completely healed in all the patients. All the patients were followed up for 12-18 mo, and no malignant gastric lesions were found.

DISCUSSION

Antigen receptor gene rearrangement in lymphocytes is a physiological process. Tumor cells that originate from lymphocytes often carry the same *Ig* and *TCR* gene rearrangements (monoclonal), whereas T and B cells have a unique type of rearrangement in benign lymphoid disorders (polyclonal). Antigen receptor gene rearrangement analysis is useful in differentiating between malignant lymphoproliferative disorders and non-neoplastic lymphoid disorders. There are several PCR targets for the detection of Ig and TCR rearrangements. Three multiplex PCR assays are available for the detection of clonal IgH (VH-JH) rearrangements, and these assays can reliably identify clonal B-cell proliferation and *TCR-γ* gene monoclonal rearrangements that occur in most T-cell lymphoid neoplasms[16]. IgH (VH-JH) and TCR-γ are the most common PCR targets for detecting Ig and TCR rearrangements.

In the PGL group, the positive rate of monoclonal *IgH* gene rearrangement in patients with stage IIE PGL was 100% but only 46.1% in patients with stage IE PGL. Previous studies have demonstrated that the positive rate of monoclonal *IgH* gene rearrangements was associated with histological grading (the histological grading of lymphoid infiltrates in the stomach according to the Wother-

spoon-Isaacson histological scoring system). Aiello *et al*[10] reported that monoclonal *IgH* gene rearrangements were detected in 64.2%, 41.6%, and 3.1% of samples with histological grading scores of 5, 4, and 0-3, respectively. Additionally, the results of this study suggest that the positive rate of monoclonal *IgH* gene rearrangements may be associated with PGL staging.

In the PGL group, 75% (12/16) of MALT lymphoma patients were positive for monoclonal IgH gene rearrangements. However, only 40% (2/5) of DLBCL patients were positive for monoclonal IgH gene rearrangements. A previous study reported that the positive rate of monoclonal *IgH* gene rearrangements in MALT lymphoma patients ranged from 62.5%-98.5%[10-13]. In the series in this study, a similar rate was observed for monoclonal *IgH* gene rearrangements in MALT lymphoma patients. Thériault *et al*[17] reported that the positive rate of monoclonal IgH gene rearrangements in DLBCL patients was 78.9% (30/38). However, the specimens in the study included lymph nodes, tonsils, spleens, bone marrow, skin biopsies, and gastrointestinal tract samples. In this study, the positive rate for DLBCL patients was lower than that in the previous study. In addition, recent studies have demonstrated that the detection rate of monoclonal *IgH* gene rearrangement was closely associated with the cell origin of lymphomas[9].

The differential diagnosis between gastric linitis plastica and PGL is not easy for a physician to determine. PGL and gastric linitis plastica usually result in low rates of positive endoscopic biopsies[18]. In this series, the first endoscopic biopsies from PGL and gastric linitis plastica patients were all negative. The distinction between PGL and gastric linitis plastica is important because of the different prognoses of these diseases. In this study, 14 (66.7%) patients in the PGL group were positive for a monoclonal *IgH* gene rearrangement; however, no IgH gene rearrangements were detected in the patients in the gastric linitis plastica group. One case of gastric cancer was positive for a monoclonal *IgH* gene rearrangement. The patient was diagnosed with carcinoma accompanied by lymphoma[12]. This result suggests that the detection of *IgH* gene rearrangements may be helpful in differentiating between PGL and gastric linitis plastica.

According to previous studies, monoclonal rearrangements involving *IgH* genes were detected in 3% of lymphoid disorders that were benign based on clinical and immunohistological evaluations, which is consistent with the findings of this study[19]. The majority of these patients suffered from autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematous, and Sjögren's syndrome. These diseases are characterized by polyclonal B-cell activation and autoantibodies[20]. In the series in this study, a monoclonal *IgH* gene rearrangement was detected in one patient who was suffering from a benign gastric ulcer. Additionally, this patient had suffered from Sjögren's syndrome for several years.

Gene rearrangement studies can be informative; however, false-positive and false-negative PCR results are problematic. According to previous studies, there are two main problems with PCR techniques: improper primer annealing and difficulties in discriminating between monoclonal and polyclonal *Ig/TCR* gene rearrangements[16,21,22]. Single-strand conformation polymorphism analysis, denaturing gradient gel electrophoresis, heteroduplex analysis, or gene scanning may be performed to reduce false-positive and false-negative rates[16,23-26].

In this study, there was one patient with false-negative results in the PGL group. The three endoscopic biopsies for this patient were negative, but the PCR results for a monoclonal *IgH* gene rearrangement were always positive. Autoimmune diseases were excluded. The patient was followed up for 7 mo and was diagnosed with PGL based on the fourth endoscopic biopsy. In addition, Fend *et al*[14] reported that the detection of clonal rearrangements in the biopsy specimens from two patients preceded the histological diagnosis of lymphoma by several mo. Because the detection of clonal rearrangements can precede a histological diagnosis, we suggest that patients with suspected PGL and positive results for IgH rearrangements need close follow-up.

In conclusion, the presence of an *IgH* gene rearrangement is useful for the diagnosis of PGL and for differentiating between PGL and gastric linitis plastica. Additionally, *IgH* gene rearrangements may be associated with PGL staging.

COMMENTS

Background

Primary gastric lymphoma (PGL) is a relatively rare tumor type. The diagnosis of PGL by gastroscopy and biopsy is often difficult. The analysis of antigen receptor gene rearrangements by polymerase chain reaction is a routine diagnostic tool for lymphoproliferative disorders. Previous studies have demonstrated that the positive rate of gene rearrangement was relatively high in PGL patients; however, no prospective studies of gene rearrangement in PGL patients have been reported.

Research frontiers

Studies are being performed to assess the diagnostic value of immunoglobulin heavy chain (*IgH*) and T-cell receptor γ (*TCR-γ*) gene monoclonal rearrangements in PGL patients.

Innovations and breakthroughs

This study was a prospective analysis of *IgH* and *TCR-γ* gene rearrangements in endoscopic biopsy specimens from patients with suspected PGL. According to the pathological results, 48 patients with suspected PGL were divided into three groups, including a PGL group, a gastric linitis plastica group, and a benign gastric ulcer group. The study revealed that the detection of monoclonal *IgH* gene rearrangements is useful for the diagnosis of PGL and for differentiating between PGL and gastric linitis plastica. Additionally, these gene rearrangements may be associated with PGL staging.

Applications

The results of this study may encourage the detection of *IgH* gene rearrangements for a diagnosis of PGL and for differentiating between PGL and gastric linitis plastica.

Terminology

Antigen receptor gene rearrangement is a physiological process. Tumor cells that originate from lymphocytes often carry the same *IgH* and *TCR* gene rearrangements (monoclonal), whereas T and B cells have a unique type of rearrangement in benign lymphoid disorders (polyclonal). Antigen receptor gene rearrangement analysis is useful in differentiating between malignant lymphoproliferative disorders and non-neoplastic lymphoid disorders.

Peer review

This paper includes interesting results and presents an acceptable case for publication because few reports have been published on this subject in China.

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BRIEF ARTICLE

Application value of multi-slice spiral computed tomography for imaging determination of metastatic lymph nodes of gastric cancer

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Abstract

AIM: To evaluate the application value of multi-slice spiral computed tomography (MSCT) for imaging determination of metastatic lymph nodes of gastric cancer and to explore reasonable diagnostic criteria.

METHODS: Sixty patients with gastric cancer underwent 64 MSCT scans before operation. Gastric cancer samples and perigastric lymph nodes were obtained after operation, formalin fixation and haematoxylin-eosin staining. The metastatic conditions of gastric cancer and perigastric lymph nodes were determined under a light microscope. A total of 605 lymph nodes were grouped and assessed according to distribution, size, shape and degree of lymph node enhancement. Then, the findings were compared with the postoperative pathological results.

RESULTS: Among 605 lymph nodes, 358 were confirmed as metastatic, accounting for 59.2%. A total of 535 lymph nodes were detected in original axis images combined with multiplanar reconstruction images of MSCT. The metastatic lymph nodes had specific signs in computed tomography. This study showed that the long diameter of lymph nodes $\geqslant$ 8 mm indicated metastasis; the sensitivity and specificity were 79.6% and 78.8%, respectively. The difference of the mean value of lymph node enhancement density $\geqslant$ 80 Hu indicated metastasis; the sensitivity and specificity were 81.6% and 75.6%, respectively. The ratio of short diameter to long diameter of lymph nodes $\geqslant$ 0.7 indicated metastasis; the sensitivity and specificity were 85.6% and 71.8%, respectively.

CONCLUSION: MSCT is a non-invasive and reliable method for preoperative examination of gastric cancer. Sensitivity and specificity for prediction of lymph node metastasis are high.

Key words: X-ray computer; Gastric cancer; Metastatic lymph nodes

Core tip: Gastric cancer is one of the most common malignant tumours of the digestive system. In recent years, individualised surgical therapy has been applied for gastric cancer. This study plan explored the distribution, size, shape and enhancement characteristics of metastatic lymph nodes. It also provided a basis for determining lymph node metastasis before surgery by retrospectively analyzing multi-slice spiral computed tomography manifestations of lymph nodes of patients with gastric cancer after surgery in the hospital. The

findings were compared with the pathological results.

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INTRODUCTION

Gastric cancer is one of the most common malignant tumours of the digestive system. In recent years, individualised surgical therapy has been applied for gastric cancer. The choice of reasonable surgical methods for different stages of gastric cancer depends on accurate preoperative diagnosis. The traditional diagnostic methods of gastric cancer include gastrofiberscope and barium meal of the upper gastrointestinal tract. However, these two methods have certain limitations in the diagnosis of gastric cancer. They cannot clearly display the gastric wall structure, and their ability to determine the presence or absence of adjacent organ invasion, distant metastasis and lymph node metastasis is limited. Multi-slice spiral computed tomography (MSCT) has been used to conduct TM preoperative staging among many patients with gastric cancer in recent years. Original axial images combined with multiplanar reconstruction (MPR) reorganised images are used to carry out preoperative assessment. The assessment includes the location, extent, depth of invasion of gastric cancer, relationship with adjacent organs and metastasis of abdominal organs. Satisfactory results have been achieved. Research on lymph node metastasis of gastric cancer before surgery has shown that a unified standard for determining lymph node metastasis by MSCT is not available worldwide. Lymph node metastasis is a major metastatic mechanism of gastric cancer. Seto *et al*[1] reported that the rate of lymph node metastasis of early gastric cancer is 5.7%-29.0%, and the rates are 0.0%-6.4% and 9.7%-24.3% for early gastric intramucosal carcinoma and gastric submucosal carcinoma, respectively. Yasuda *et al*[2] showed that the rate of lymph node metastasis of early gastric cancer is 8.9%, and the rates are 2.5% and 17.6% for early gastric intramucosal carcinoma and gastric submucosal carcinoma, respectively. Okusa *et al*[3] proposed the concept of metastatic lymph node ratio (MLR). The results of numerous studies worldwide[4,5] have shown that MLR is one of the independent prognostic factors of survival of patients with gastric cancer, which is closely correlated with the five-year survival rate of patients with gastric cancer. Both the Union for International Cancer Control (UICC) and the Japanese General Rules for Gastric Cancer Study consider that lymph node metastasis is an independent and important factor for predicting the prognosis of patients with gastric cancer[6,7]. To date, radical gastrectomy of gastric cancer with radical lymph node excision adjacent to the stomach has achieved better therapeutic effects in the surgical therapy of gastric cancer[8]. The presence or absence of lymph node metastasis, as well as the degree and extent of metastasis, is directly correlated with the choice of therapeutic methods and prognostic evaluation of patients with gastric cancer, which are primary indices for surgical approach selection. Radical excision of metastatic lymph nodes has important clinical significance and prognostic value in patients with gastric cancer. Radical excision of metastatic lymph nodes may affect the immune function in patients and lead to increased surgical trauma, which cannot improve curative effect. Therefore, confirming the presence or absence of lymph node metastasis of gastric cancer before surgery is important for preoperative staging, formulation of clinical therapeutic schedule and prognosis evaluation[9]. MSCT has the advantages of rapid scanning speed, high resolution ratio and convenient image reconstruction, which can estimate lymph nodes by thin-layer scanning and reconstruction technique and direct clinical staging[10-12]. The standard for determining metastasis of lymph nodes by enhancement characteristics and lymph node size displayed by MSCT is the focus of studies and controversies among many researchers. Too high or too low MSCT staging may appear during clinical application because of different standards of size and morphology of lymph nodes and different sizes of lymph nodes in different positions[13]. Thus, this study plan explored the distribution, size, shape and enhancement characteristics of metastatic lymph nodes. It also provided a basis for determining lymph node metastasis before surgery by retrospectively analyzing MSCT manifestations of lymph nodes of patients with gastric cancer after surgery in the hospital. The findings were compared with the pathological results.

MATERIALS AND METHODS

Objectives

Sixty patients with gastric cancer who were hospitalised in the First Affiliated Hospital of Jilin University and underwent MSCT scanning before operation from February 2010 to October 2011 were included the study. The patients comprised 48 male and 12 female with a mean age of 59.5 years (36-78 years). A total of 51 patients were confirmed to have metastatic lymph nodes of gastric cancer after operation, whereas no metastatic lymph nodes were observed in nine patients. The histopathological types included poorly differentiated adenocarcinoma (39 patients), moderately differentiated tubular adenocarcinoma (17 patients) and signet-ring cell carcinoma (4 patients).

Methods

All the patients were asked to fast for 6-8 h before scanning. They were treated with intramuscular injection of 654-2 (20 mg) and oral administration of warm water 10 min before scanning. The patients were scanned in

supine position using 64-MSCT (Siemens, Germany). Scanning parameters were as follows: spiral collimation, 64 × 0.625; thickness of every layer, 5 mm; interval thickness of every layer, 5 mm; speed of bed movement, 12 mm/s; tube voltage, 120 kV and tube current, 260-320 mAs. During plain, arterial and venous scanning phases, the extent of scanning was from the lower oesophagus to the level of inferior pole of kidney, including the whole gastric area. During equilibrium phase, the extent of scanning was from the diaphragmatic dome to the pelvic cavity. Each scan was performed during a breath hold at the end of inspiration. Anconal venous transfusion of non-ionic contrast medium (OmniPaque 300 or Ultravist 300; 80-100 mL) with high pressure injector was used during the enhanced scanning. The rate of injection was 3.0 mL/s, and the starting times of arterial scanning, venous scanning and equilibrium phases were 25, 35 and 60 s after the beginning of injection. After scanning, the original data were treated with thin-slice reconstruction (1 mm; the interval between two adjacent slices was 1 mm). The images of all patients underwent MPR.

Image analysis

Two doctors with years of experience on abdominal image diagnosis analysed and treated the images. According to anatomic sites, original axis images combined with MPR were applied to observe various indices of lymph nodes, including distribution, number, size, shape and degree of lymph node enhancement.

Evaluation of results

Evaluation criteria of CT signs: The lymph nodes were divided into three groups according to the long diameter: ≥ 5 mm group, ≥ 8 mm group and ≥ 10 mm group. Ten points of non-cystic area in each detected lymph node were randomly selected in MSCT. The mean difference of CT value during venous scanning phase and plain scanning was measured. Then, the lymph nodes were divided into three groups according to the degree of enhancement: the difference of mean value of enhancement density ≥ 100 Hu group, ≥ 80 Hu group and ≥ 40 Hu group. The lymph nodes were divided into two groups according to the ratio of short diameter to long diameter: ≥ 0.5 group and ≥ 0.7 group.

Pathological criteria

Gastric cancer samples and perigastric lymph nodes were obtained after operation, formalin fixation and haematoxylin-eosin staining. The metastatic conditions of gastric cancer and perigastric lymph nodes were determined under a light microscope.

Statistical analysis

All the data were analysed using SPSS17.0 software. *K* test was used to evaluate the consistency of metastatic lymph nodes of gastric cancer between MSCT and postoperative pathological diagnosis. Kappa coefficient within 0.71-1.00, 0.41-0.70 and ≤ 0.4 indicated strong, general and weak consistencies, respectively.

RESULTS

Comparison of different long diameters of lymph nodes displayed by MSCT and postoperative pathological results for determination of lymph node metastasis.

A total of 605 lymph nodes were cleaned up in the operation, among which 358 were diagnosed as metastatic by postoperative pathological examination, accounting for 59.2%. Original axis images combined with MPR images found 535 lymph nodes. The lymph nodes could be analysed and determined with a group of lymph nodes as a unit in MSCT images because the lymph nodes were excised with a group as a unit instead of surgically operated, according to the images. A single lymph node could not be specifically studied. Thus, the ability of MSCT to determine the specificity and sensitivity of lymph nodes could be improved as a whole in the study. The data from the different groups were compared with the pathological results. The consistency of lymph node diameter ≥ 5 mm and the postoperative pathological results was general ($K = 0.464$). The consistency of lymph node diameter ≥ 8 mm and the postoperative pathological results was strong ($K = 0.831$). The consistency of lymph node diameter ≥ 10 mm and the postoperative pathological results was weak ($K = 0.232$) (Table 1).

Comparison of different degrees of lymph node enhancement displayed by MSCT and postoperative pathological results for determination of lymph node metastasis.

The consistency of the difference of the mean value of enhancement density ≥ 80 Hu and the postoperative pathological results was strong ($K = 0.849$). The consistency of the difference of the mean value of enhancement density ≥ 100 Hu and the postoperative pathological results was weak. The consistency of the difference of the mean value of enhancement density ≥ 40 Hu and the postoperative pathological results was weak ($K < 0.40$) (Table 1).

Comparison of different ratios of short diameter to long diameter of lymph nodes displayed by MSCT and postoperative pathological results for determination of lymph node metastasis.

The consistency of the ratio of short diameter to long diameter of lymph nodes ≥ 0.7 and the postoperative pathological results was strong ($K = 0.873$). The consistency of the ratio of short diameter to long diameter of lymph nodes > 0.5 and the postoperative pathological results was general ($K = 0.513$) (Table 1).

The diagnostic criteria of metastatic lymph nodes in MSCT for patients with gastric cancer included long diameter of lymph nodes ≥ 8 mm, the ratio of short diameter to long diameter of lymph nodes ≥ 0.7 and the difference of the mean value of enhancement density ≥ 80 Hu. Compared with the postoperative pathological results, the sensitivities and specificities of MSCT for detection of lymph nodes adjacent to the celiac artery and lesser curvature were 89.4% and 90.3%, respectively. The

Table 1 Comparison of different long diameters of lymph nodes displayed, degrees of lymph node enhancement displayed and ratios of short diameter to long diameter of lymph nodes displayed by multi-slice spiral computed tomography and postoperative pathological results for determination of lymph node metastasis

		Number of lymph nodes detected by MSCT	*K* value	Sensitivity	Specificity
Diameter of lymph nodes	≥ 5 mm	470	0.464	88.5%	60.1%
	≥ 8 mm	317	0.831	79.6%	78.8%
	≥ 10 mm	128	0.232	48.6%	93.5%
Difference of enhancement of lymph nodes	≥ 40 Hu	495	0.397	89.3%	65.5%
	≥ 80 Hu	379	0.849	81.6%	75.6%
	≥ 100 Hu	197	0.335	53.8%	95.5%
Ratio of short diameter to long diameter of lymph nodes	≥ 0.5	447	0.513	94.3%	57.3%
	≥ 0.7	375	0.873	85.6%	71.8%

MSCT: Multi-slice spiral computed tomography.

Table 2 Comparison of sensitivities and specificities of metastatic lymph nodes in different groups displayed by multi-slice spiral computed tomography

Positions of lymph nodes	Specificity	Sensitivity
Right area of cardiac orifice	79.2%	57.8%
Left area of cardiac orifice	81.3%	73.5%
Lesser curvature	79.6%	90.3%
Greater curvature	87.5%	45.0%
Superior area of pylorus	88.1%	76.7%
Inferior area of pylorus	78.0%	81.9%
Adjacent to left gastric artery, common hepatic artery, and arteria coeliaca	83.2%	89.4%

detection rates of lymph nodes in the right area of the cardiac orifice and adjacent to the greater curvature were 57.8% and 45.0%, respectively (Table 2).

DISCUSSION

Tumour-node-metastasis staging system is one of the most commonly used staging systems, and is accepted and maintained by the UICC and the American Joint Committee on Cancer[14]. Lymph node metastasis is a major metastatic pathway of gastric cancer. The metastatic rates of lymph nodes in gastric cancer at early and progressive stages are 10% and 74.8%, respectively[15]. The lymph node size determines the lymph node metastasis. Lymph node diameter > 10 mm is used as one of the criteria to diagnose lymph node metastasis of gastric cancer by MSCT. Some researchers have suggested that the lymph node diameter > 5 mm can be used as a criterion of lymph node metastasis of gastric cancer[16,17]. Dux proposed that all the lymph nodes detected by MSCT could be considered as lymph node metastasis. Some researchers have considered that the short diameter of perigastric lymph nodes > 6 mm or the short diameter of lymph nodes adjacent to the stomach > 8 mm should be regarded as metastasis[18]. Moreover, other researchers believe that determining lymph node metastasis by imaging is not sufficient because the sensitivity of revealing small lymph nodes by imaging is low. This study selected 5, 8 and 10 mm (long diameter of lymph nodes) as threshold values of lymph node metastasis, and then a comparative study was performed. The long lymph node diameter of 8 mm was determined as the threshold value of lymph node metastasis after statistical analysis. Compared with the postoperative pathological results, the sensitivity and specificity were more reasonable.

The degree of lymph node enhancement is an important index to determine lymph node metastasis. The perigastric lymph nodes have a specific blood supply, and this blood supply is abundant when metastasis of lymph nodes occurs. After MSCT enhancement, obvious lymph node enhancement is displayed, but the non-metastatic lymph nodes exhibit absence of enhancement or mild enhancement. Fukuya *et al*[19] posited that high density or peripheral high density and central low density of metastatic lymph nodes of gastric cancer show moderate or obvious enhancement, but non-metastatic lymph nodes show no enhancement or mild enhancement. Some researchers believe that CT value ≥ 25 Hu during plain scanning phase, CT value ≥ 70 Hu during arterial scanning phase or CT value ≥ 80 Hu during venous scanning phase is a criterion of positive lymph nodes, which can significantly improve the diagnostic rate of lymph node metastasis[20,21]. In this study, the consistency of the difference of the mean value of enhancement density ≥ 80 Hu and the postoperative pathological results was strong (K = 0.849). The sensitivity and specificity were 81.6% and 75.6%, respectively. Selecting 80 Hu is recommended to determine the threshold value of metastatic lymph nodes of gastric cancer.

The morphology of lymph nodes displayed by MSCT is also an important index to determine lymph node metastasis. Certain exogenous and expansible growth characteristics are shown in lymph nodes when lymph node metastasis occurs. These characteristics contribute to the round or oval morphology of lymph nodes, and the expansible growth of lymph nodes is not balanced so the margin of metastatic lymph nodes is irregular and appears blurred. Thus, the morphology of lymph nodes is one of the references in determining lymph node metastasis by imaging. Fukuya *et al*[19] posited that the ratio of short diameter to long diameter of lymph node metastasis of gastric cancer was (0.81 ± 0.15), but the ratio of short diameter to long diameter of lymph node metastasis of gastric cancer was (0.57 ± 0.15). This study showed

that the consistency of the ratio of short diameter to long diameter of lymph nodes ≥ 0.7 and the postoperative pathological results was strong (K = 0.873). When the ratio of short diameter to long diameter of lymph node metastasis of gastric cancer ≥ 0.7 was selected as a criterion, the sensitivity and specificity were 85.6% and 71.8%, respectively. This result showed that the ratio of short diameter to long diameter of lymph node metastasis of gastric cancer ≥ 0.7 in MSCT is more reasonable as a criterion.

The detection rate of lymph nodes in MSCT is correlated with the location of lymph nodes. Some studies have reported that the detection rates of MSCT for mesenteric lymph nodes and lymph nodes adjacent to the aorta were the highest, followed by the lymph nodes adjacent to the lesser curvature and celiac artery. The detection rates of MSCT for lymph nodes adjacent to the ligamentum hepatoduodenale and common hepatic artery were relatively low, and the detection rate of MSCT for lymph nodes adjacent to the nidi was low[6]. The statistical results of the study showed that the sensitivities of MSCT for lymph nodes adjacent to the celiac artery and lesser curvature were 89.4% and 90.3%, respectively. However, the detection rates of MSCT for lymph nodes adjacent to the right area of cardiac orifice and greater curvature were relatively low, especially the lymph nodes adjacent to the greater curvature (45.0%). These results were attributed to the lymph nodes adjacent to the lesser curvature, which were near the stomach wall. The lymph nodes showed soft tissue density; after filling the stomach with water, the lymph nodes were easily foiled. The lymph nodes adjacent to the celiac artery were near the vessels, and lymph node enhancement and vascular enhancement were not significantly co-gradient. They were easily distinguished according to density; thus, the sensitivity of lymph nodes adjacent to the celiac artery was high. The perigastric fat content is one of the important factors affecting the detection rate of lymph nodes by MSCT. In this study, the stomachs of the patients were filled after drinking water, thereby reducing the fat adjacent to the greater curvature and surrounding organs. This phenomenon caused the surrounding lymph nodes not to be displayed, which was one of the main reasons for the low detection rate of lymph nodes adjacent to the greater curvature. In addition, the resolution ratio of MSCT for lymph nodes adjacent to the pancreas was relatively low because most of the patients with gastric cancer were elderly. Their pancreas atrophied, showing nodositas. The densities of lymph nodes adjacent to the pancreas during plain scanning and enhancement scanning were similar to the density of pancreatic substance. The lymph nodes were easily diagnosed as swelling lymph nodes, so determining swelling lymph nodes adjacent to the pancreas by MSCT has a certain limitation.

In conclusion, conducting MSCT examination before operation among patients with gastric cancer, as well as observing and measuring the size, degree of enhancement and morphology of lymph nodes, is helpful in determining lymph node metastasis before operation. The results will provide references for staging of gastric cancer before operation and for choosing a therapeutic schedule.

COMMENTS

Background

Lymph node metastasis is the most important metastatic methods of gastric cancer; it is also one of the important factors affecting the prognosis of patients. The presence or absence of lymph node metastasis, as well as the degree and extent of metastasis, is directly correlated with the choice of therapeutic methods and prognostic evaluation of gastric cancer. Considerable studies exist on predicting lymph node metastasis of gastric cancer before surgery by multi-slice spiral computed tomography (MSCT). A number of predictive criteria are available, including distribution, size and enhancement of lymph nodes, but they are not unified and acknowledged diagnostic criteria.

Research frontiers

MSCT is widely used for diagnosis of preoperative staging of gastric cancer, especially 64-row MSCT or above. This method significantly improves the resolution ratio of images, achieves complete accordance of resolution ratios on axial view, coronal view, sagittal view, inclined plane and curved surface, and provides valuable information for displaying metastatic lymph nodes.

Innovations and breakthroughs

This study investigated the enhancement characteristics of metastatic lymph nodes displayed by MSCT and the relationship between the size of lymph nodes and metastasis. This research also determined the effects of different positions of lymph nodes on the detection rate of MSCT and the relationship between morphology of lymph nodes and metastasis. Preoperative MSCT scanning is one of the important tools used to evaluate the prognosis of patients with gastric cancer.

Applications

N-stage and prognostic evaluation of gastric cancer were performed and applied in the First Affiliated Hospital of Jilin University according to the diagnostic criteria of lymph node metastasis of gastric cancer before surgery designed in this study. The accurate rate of preoperative MSCT for evaluation of lymph node metastasis of gastric cancer was high based on the pathological results of more than 60 patients with gastric cancer after surgery.

Peer review

Application value of multi-slice spiral computed tomography for imaging determination of metastatic lymph nodes of gastric cancer. In this article, authors try to evaluate the application value of multi-slice spiral computed tomography for imaging determination of metastatic lymph nodes of gastric cancer and to explore reasonable diagnostic criteria. As is known, MSCT has been used to conduct TM preoperative staging among many patients with gastric cancer in recent years.

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META-ANALYSIS

Induction of clinical response and remission of inflammatory bowel disease by use of herbal medicines: A meta-analysis

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Abstract

AIM: To evaluate the efficacy and tolerability of herbal medicines in inflammatory bowel disease (IBD) by conducting a meta-analysis.

METHODS: Electronic databases were searched for studies investigating efficacy and/or tolerability of herbal medicines in the management of different types of IBD. The search terms were: "herb" or "plant" or "herbal" and "inflammatory bowel disease". Data were collected from 1966 to 2013 (up to Feb). The "clinical response", "clinical remission", "endoscopic response", "endoscopic remission", "histological response", "histological remission", "relapse", "any adverse events", and "serious adverse events" were the key outcomes of interest. We used the Mantel-Haenszel, Rothman-Boice method for fixed effects and DerSimonian-Laird method for random-effects. For subgroup analyses, we separated the studies by type of IBD and type of herbal medicine to determine confounding factors and reliability.

RESULTS: Seven placebo controlled clinical trials met our criteria and were included (474 patients). Comparison of herbal medicine with placebo yielded a significant RR of 2.07 (95%CI: 1.41-3.03, P = 0.0002) for clinical remission; a significant RR of 2.59 (95%CI: 1.24-5.42, P = 0.01) for clinical response; a non-significant RR of 1.33 (95%CI: 0.93-1.9, P = 0.12) for endoscopic remission; a non-significant RR of 1.69 (95%CI: 0.69-5.04) for endoscopic response; a non-significant RR of 0.64 (95%CI: 0.25-1.81) for histological remission; a non-significant RR of 0.86 (95%CI: 0.55-1.55) for histological response; a non-significant RR of 0.95 (95%CI: 0.52-1.73) for relapse; a non-significant RR of 0.89 (95%CI: 0.75-1.06, P = 0.2) for any adverse events; and a non-significant RR of 0.97 (95%CI: 0.37-2.56, P = 0.96) for serious adverse events.

CONCLUSION: The results showed that herbal medicines may safely induce clinical response and remission in patients with IBD without significant effects on endoscopic and histological outcomes, but the number of studies is limited to make a strong conclusion.

Key words: Herbal medicine; Inflammatory bowel disease; Efficacy; Relapse; Adverse events; Meta-analysis

Core tip: Meta-analysis of seven controlled trials involving 474 patients demonstrated that herbal medicines may safely induce clinical response and remission in patients with inflammatory bowel disease without significant effects on endoscopic and histological outcomes. The results of sub-analyses based on plant

type demonstrated that induction of clinical remission was obtained only by *Artemisia absinthium* and *Boswellia serrata* and induction of clinical response was gained by only *Aloe vera* and *Triticum Aestivum*. *Boswellia serrata* in one study evaluating recurrence rate did not cause prevention of relapse. Induction of adverse events by none of the plants was significant compared to that of placebo.

Rahimi R, Nikfar S, Abdollahi M. Induction of clinical response and remission of inflammatory bowel disease by use of herbal medicines: A meta-analysis. *World J Gastroenterol* 2013; 19(34): 5738-5749 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i34/5738.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i34.5738

INTRODUCTION

Inflammatory bowel disease (IBD) is a group of inflammatory conditions of gastrointestinal tract with two major types including ulcerative colitis (UC) and Crohn's disease (CD) and some atypical forms like collagenous colitis and intractable colitis. Many etiological factors have been implicated to play role in IBD; the most important one is immunological disturbances. Different drug categories are used for the management of IBD like aminosalicylates[1], corticosteroids[2], anti-tumor necrosis factor alpha drugs[3,4], antibiotics[5,6], probiotics[7,8], and immunosuppressants[9]. Because of lack of desirable efficacy and poor tolerability of these drugs, approach toward complementary and alternative medicines especially herbal medicines for the management of IBD are increasing[10,11]. Besides many *in vivo* studies[12-14], the efficacy and tolerability of herbal medicines in IBD have been investigated through several clinical trials. In this paper, all of these clinical trials were retrieved and a meta-analysis was performed to obtain conclusive results about efficacy and tolerability of herbal medicines for the management of IBD.

MATERIALS AND METHODS

Methods

The procedures performed in this meta-analysis are in accordance with recent guidelines for the reporting of meta-analysis (PRISMA guidelines).

Data sources and searches

PubMed, Scopus, Web of Science, and Cochrane Central Register of Controlled Trials were searched for studies evaluating efficacy and/or tolerability of herbal medicines in any types of IBD. Data were collected from 1966 to 2013 (up to Feb). The search terms were: "herb" or "plant" or "herbal" and "inflammatory bowel disease". There was no language restriction. The reference list from retrieved articles was also reviewed for additional applicable studies.

Study selection

Controlled trials evaluating the efficacy and/or tolerability of herbal medicines in patients with any types of IBD were considered. The "clinical response", "clinical remission", "endoscopic response", "endoscopic remission", "histological response", "histological remission", "relapse", "any adverse events", and "serious adverse events" were the key outcomes of interest. All published studies as well as abstracts presented at meetings were evaluated. Two reviewers independently examined the title and abstract of each article to eliminate duplicates, reviews, case studies, and uncontrolled trials.

The reviewers independently extracted data on patients' characteristics, therapeutic regimens, dosage, trial duration, and outcome measures. There was no disagreement between reviewers.

Quality assessment

Jadad score, which indicates the quality of the studies based on their description of randomization, blinding, and dropouts (withdrawals) was used to assess the methodological quality of trials[15]. The quality scale ranges from 0 to 5 points with a low quality report of score 2 or less and a high quality report of score at least 3.

Statistical analysis

Data from selected studies were extracted in the form of 2 × 2 tables by study characteristics. Included studies were weighted and pooled. Data were analyzed using StatsDirect software version 2.7.9. RR and 95%CI were calculated using Mantel-Haenszel, Rothman-Boice (for fixed effects) or Der Simonian-Laird (for random effects) methods. The Cochran Q test was used to test heterogeneity and $P < 0.05$ considered significant. In case of heterogeneity or few included studies, the random effects model was used. Funnel plot was used as publication bias indicator.

RESULTS

The electronic searches yielded 1224 items; 698 from PubMed, 5 from Cochrane Central, 35 from Web of Science, and 355 from Scopus. Of those, 41 trials were scrutinized in full text.

Thirty four reports were considered ineligible. Thus, 7 trials were included in the analysis represented 474 patients (Figure 1)[16-22]. From these 7 studies, 5 obtained Jadad score of 4 or more[16,17,20-22] and remaining two gained Jadad score of 2[18,19] (Table 1). Among studies included, 3 investigated the efficacy and/or tolerability of herbal medicines in CD[18-20], 3 in UC[16,17,22] and 1 in collagenous colitis[21]. Five plants were investigated in 7 included studies: *Aloe vera*[16], *Andrographis paniculata*[17], *Artemisia absinthium*[18,19], and *Boswellia serrata*[20,21], and *Triticum aestivum*[22]. Induction of treatment was investigated in six studies and duration of these studies is between 4

Table 1 Characteristics of studies included in the meta-analysis

Study	Scientific name of plant(s)	Study design	Method of randomization	Blindness	Withdrawal	Jadad score	Inclusion criteria	Exclusion criteria	Interventions	Concomitant medications	Duration	Outcomes
Sandborn *et al*[17]	*Andrographis paniculata*	Randomized, placebo-controlled, double-blind	Block randomization schedule	Double-blind	32 patients in *Andrographis* group and 11 in placebo group	4	Patients with at least 18 yr of age and confirmed diagnosis of mildly to moderately active UC (Mayo Score of 4-10 points and endoscopic subscore of at least 1) while receiving either oral mesalamine (or equivalent medications sulfasalazine, balsalazide, and olsalazine) for at least 4 wk or no medical therapy	Patients with CD or indeterminate colitis, severe UC (Mayo Score of 11 or 12 points, toxic mega-colon, toxic colitis), previous colonic surgery or probable requirement for intestinal surgery within 12 wk, enteric infection within 2 wk, a history of tuberculosis, a positive chest X-ray or tuberculin protein-purified derivative skin test, active infection with hepatitis B or any infection with hepatitis C, infection with human immunodeficiency virus, cancer within 5 yr, inadequate bone marrow, hepatic, or renal function, a history of alcohol or drug abuse that would interfere with the study, significant concurrent medical diseases, allergy to plants in the Acanthacea family, women who were pregnant or breastfeeding , receiving oral or rectal steroids within 1 mo, rectal mesalamine within 1 wk, antibiotics within 2 wk, or azathioprine, 6-mercaptopurine, anti-tumor necrosis factor agents, or immunosuppressive therapy within 6 wk	Group 1: Capsules containing 1200 or 1800 mg *Andrographis paniculata* ethanol extract. [n = 149 (male/female: 81/68)]. 1 cap *tds* Group 2: The same capsules without herbal extract. [n = 75 (male/female: 41/34)]. 1 cap *tds*	Mesalazine	8 wk	(1) Clinical response (a decrease from baseline in the total Mayo Score by at least 3 points and at least 30% with an accompanying decrease in rectal bleeding subscore of at least 1 point or a absolute rectal bleeding subscore of 0 or 1 point); (2) Clinical remission (a total Mayo Score of 2 points or lower, with no individual subscore exceeding 1 point); (3) Mucosal healing (a decrease from baseline in the endoscopy subscore by at least 1 point and an absolute endoscopy subscore of 0 or 1 point)
Holtmeier *et al*[20]	*Boswellia serrata*	Randomized, placebo-controlled, double-blind	A computer generated randomization scheme: In blocks of four	Double-blind	9 patients in *Boswellia* group and 7 in control group	4	Outpatients between 18 and 75 yr with a history of CD currently in remission with at least two documented relapses during the last 4 yr, one within the last 18 mo, or a recent resection (fibrotic strictures without inflammation were not considered a relapse); CDAI < 150 and no symptoms suspicious of activity for the previous 28 d	CDAI of > 150 at screening and at baseline visit (≥ 28 d apart); severe fistulizing CD; abscesses; symptomatic stenoses; any condition that places the patient at an undue risk; surgical bowel resections within 3 mo, short bowel syndrome; total proctocolectomy; serious infections, nutritionally compromised patients requiring enteral or parenteral therapy; severe hypertension, chronic liver disorder; impaired renal function; myocardial infarction < 3 mo, cerebral blood flow disturbances or cerebral infarction < 6 mo; any history of malignancy within the past 5 yr (except for squamous or basal cell carcinoma of the skin); subjects with severe psychiatric illnesses, inability to give informed consent; and history of severe alcoholism and drug abuse; taken monoclonal antibody therapy (*e.g.*, infliximab) within 12 mo, immunosuppressives (azathioprine/6-mercaptopurine, cyclosporine, methotrexate) within 4 mo, or corticosteroids, mesalamine/sulfasalazine, or Boswellia serrata within 6 wk prior to randomization	Group 1: Capsules containing 400 mg 8% ethanol extract of *Boswellia serrata* resin. [n = 42 (male/female: 13/29)]. 2 caps *tds* Group 2: The same capsules without herbal extract. [n = 40 (male/female: 15/25)]. 2 caps *tds*	ND	52 wk	(1) Maintenance of remission (maintenance of CDAI < 150 throughout study); (2) Relapse (relapse was defined as both a CDAI score > 150 points and an increase in the CDAI score of ≥ 70 points)

Krebs *et al*[18]	*Artemisia absinthium*	Randomized, open label	ND	Unblinded	Not any	2	Patients between 18 and 80 yr with CDAI ⩾ 200 at least for 3 mo receiving CD treatments with 5-aminosalicylates stable dose for at least 4 wk, azathioprine stable dose for 8 wk, methotrexate stable dose for 6 wk or steroids with stable dose in the range of 20-30 mg (equivalent to dexamethasone)	Treatment with TNF-α inhibitors such as infliximab; Patients with serious pathological findings in ECG, liver, kidney and heart functions, or coexisting organic diseases such as a history of cancer, asthma or other autoimmune disease, or pregnancy; any condition that in the investigators opinion placed the patient at undue risk by participating in the study; parasites in the patient's stools, positive *Clostridium difficile* toxin test and active fungal or viral infection	Group 1: Capsules containing 250 mg leave and stem powder of Artemisia bsinthium. [*n* = 10 (male/female: 6/4)]. 3 caps *tds* Group 2: No medication. [*n* = 10 (male/female: 3/7)]	Azathioprine, mesalazine	6 wk	Response: a decrease in the CDAI score of at least 70 points from the qualifying score, or a decrease in 30% of CDAI score from the baseline score
Madisch *et al*[21]	*Boswellia serrata*	Randomized, placebo-controlled, double-blind	Groups of four patients according to a central computer-generated randomization list	Double-blind	5 patients in *Boswellia* group	5	Patients, aged between 18 and 80 yr were eligible for the study if they had at least five liquid or soft stools per day on average per week, a complete colonoscopy performed within the last 4 wk before randomization, and a histologically confirmed diagnosis of collagenous colitis	Treatment with budesonide, salicylates, steroids, prokinetics, antibiotics, ketoconazole, or non-steroidal anti-inflammatory drugs within 4 wk before randomization, other endoscopically or histologically verified causes for diarrhea, infectious diarrhea, pregnancy or lactation, previous colonic surgery, and known intolerance to *Boswellia* extract	Group 1: 400 mg capsules containing boswellia serrata extract standardized to 80% boswellic acids, 1 capsule *tid* Group 2: Identical placebo capsules, 1 capsule *tid*	Loperamide was allowed for the first 3 wk but was not allowed for the last 3 wk of the study. Patients were allowed to use butylscopolamine in case of abdominal pain	6 wk	Clinical remission (stool frequency equal to or less than three soft or solid stools per day on average during the last week of treatment)
Omer *et al*[19]	*Artemisia absinthium*	Double-blind, placebo-controlled	ND	Double-blind	ND	2	Patients between 18 and 80 yr with CDAI ⩾ 200 at least for 3 mo receiving CD treatments with 5-aminosalicylates stable dose for at least 4 wk, azathioprine stable dose for 8 wk, methotrexate stable dose for 6 wk or corticosteroids (prednisolone, prednisone or budesonide) at the equivalent of 40 mg/d of prednisone or Less stable dose for 3 wk	Treatment with infliximab; patients with serious pathological findings in ECG, liver, kidney and heart functions, or coexisting organic diseases such as a history of cancer, asthma or other autoimmune disease, or pregnancy; any condition that in the investigators opinion placed the patient at undue risk by participating in the study; parasites in the patient's stools, positive *Clostridium difficile* toxin test and active fungal or viral infection	Group 1: Capsules containing 250 mg leave and stem powder of *Artemisia bsinthium*. [*n* = 20 (male/female: 12/8)]. 3 caps *bid* Group 2: The same capsules without *Artemisia absinthium*. [*n* = 20 (male/female: 11/9)]. 3 caps *bid*	Glucocorticoids, 5-aminosalicylates, azathioprines, methotrexate	10 wk	A decrease in the CDAI score of at least 70 points from the qualifying score, or a decrease in 30% of CDAI score from the baseline score

Langmead *et al*[16]	*Aloe vera*	Randomized, double-blind, placebo-controlled	Computer-generated, block-design, in 2:1 ratio	Double-blind	6 patients in aloe group and 3 in the placebo group	4	Age of 18-80 yr, mildly to moderately active UC (as defined by a modified SCCAI ⩾ 3) and no recent changes in conventional prophylactic therapy	Acute severe UC requiring hospital admission (SCCAI > 12); inactive disease (SCCAI < 3); positive stool examination for pathogens; CD or indeterminate colitis; use of antibiotics, warfarin, cholestyramine, sucralfate, anti-diarrhoeal drugs (loperamide, codeine phosphate, diphenoxylate), non-steroidal anti-inflammatory drugs, aspirin > 75 mg/d, aloe vera or other herbal remedies; alcohol or drug abuse; pregnancy or breast feeding; female of child-bearing age not taking adequate contraception; participation in another drug trial in the previous 3 mo; and serious liver, renal, cardiac, respiratory, endocrine, neurological or psychiatric illness, alteration in their dosage of aminosalicylates in the previous 4 wk, had taken > 10 mg/d or had altered oral prednisolone dosage in the previous 4 wk, changed their dose of azathioprine or 6-mercaptopurine in the previous 3 mo, or had used more than five corticosteroid or aminosalicylate enemas in the previous 2 wk	Group 1: *Aloe vera* gel. [*n* = 30 (male/female: 16/14)]. 100 mL *bid* Group 2: The same lipqiud product without *Aloe vera* gel. [*n* = 14 (male/female: 6/8)]	5-ASA, prednisolone, azathioprine, topical 5-ASA, topical steroid	4 wk	(1) Clinical remission (SCCAI≤2); (2) Sigmoidoscopic remission [Baron score of zero (normal-looking mucosa) or one (mucosal oedema as indicated by loss of the normal vascular pattern)]; (3) Histological remission (Savery-muttu score of ⩽ 1, *i.e.*, no loss of colonocytes, absence of crypt inflammation, and normal lamina propria content of mononuclear cells and neutrophils); (4) Clinical improvement (a reduction in SCCAI of ⩾ 3 points); (5) Clinical response (remission or improvement); (6) Sigmoidoscopic improvement (decrease in Baron score of ⩾ 2 points; and (7) Histological improvement (decrease in Savery-muttu score of ⩾ 3 points)
Ben-Arye *et al*[22]	*Triticum aestivum*	Randomized, double-blind, placebo-controlled	ND	Double blind; both the true juice and the placebo were packaged in coded, identical, sealed, opaque containers. A driver, blinded to the allocation scheme and given only the addresses for each package, then distributed all the packages	2 patients in triticum group and 1 in the placebo group	4	Age > 18 yr; sigmoidoscopic finding of active UC that involves the left colon; clinical activity comparable with UC; no change in drug treatment (type and dosage) in the month prior to entry; lack of serious systemic involvement–fever > 38 ℃, erythema nodosum, arthritis; blood hemoglobin > 11 g%; negative stool culture and test for ova and parasites	ND	Group 1: 100 mL of *Triticum aestivum* seed juice. [*n* = 11 (male/female: 6/5)] Group 2: 100 mL of matching placebo. [*n* = 12 (male/female: 9/3)]	-	1 mo	Improvement (larger than 0.4 in an analog scale where -3 designates the lowest score of aggravation, 0 no change, and +3 highest score of improvement)

CD: Crohn's disease; CDAI: Crohn's disease activity index; ND: Not determined; SCCAI: Simple clinical colitis activity index; UC: Ulcerative colitis; ASA: Aminosalicylic acid. ECG: Electrocardiography; TNF: Tumor necrosis factor.

Table 2 Results for outcomes investigated for each included studies

Herbal product	IBD type	Study	Patients reported AE		Clinical efficacy		Endoscopic efficacy		Histological efficacy		Recurrence relapse
			Any AE	Serious AE	Clinical remission	Clinical response	Endoscopic remission	Endoscopic response	Histological remission	Histological response	
Aloe vera	UC	16	H: 6/30 C: 4/14	-	H: 9/ 30 C: 1/14	H: 14/30 C: 2/14	H: 7/26 C: 2/11	H: 12/26 C: 3/11	H: 6/21 C: 4/9	H: 14/21 C: 7/9	-
Andrographis paniculata	UC	17	H: 84/149 C: 45/75	H: 4/149 C: 2/75	H: 53/148 C: 19/75	H: 78/148 C: 30/75	H: 65/148 C: 25/75	-	-	-	-
Artemisia absinthium	CD	18	-	H: 0/10 C: 0/10	-	H: 8/10 C: 2/10	-	-	-	-	-
Artemisia absinthium	CD	19	-	-	H: 13/20 C: 0/20	H: 18/20 C: 0/20	-	-	-	-	-
Boswellia serrata	CD	20	H: 29/42 C: 34/40	H: 4/42 C: 4/40	-	-	-	-	-	-	H: 14/42 C: 14/40
Boswellia serrata	Collagenous colitis	21	H: 2/16 C: 1/15	H: 0/16 C: 0/15	H: 10/16 C: 4/15	-	-	-	-	-	-
Triticum aestivum	UC	22	-	-	-	H: 10/11 C: 5/12	-	-	-	-	-

AE: Adverse event; C: Control; CD: Crohn's disease; H: Herbal product; UC: Ulcerative colitis; IBD: Inflammatory bowel disease.

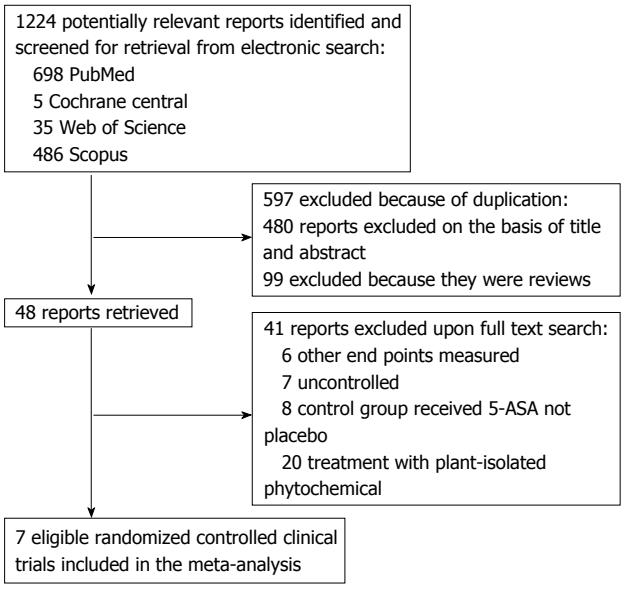

Figure 1 Flow diagram of the study selection process. ASA: Aminosalicylic acid.

and 10 wk[16-19,21,22]. Maintenance of remission was evaluated in one study and duration of this study was 52 wk[20]. Scientific name of plant(s) used in herbal medicine, study design, inclusion and exclusion criteria, interventions, concomitant medications, patients' characteristics, duration of study and definition of outcomes investigated in each included study have been shown in Table 1. Results of investigated outcomes for each included study have been demonstrated in Table 2.

Efficacy

Clinical remission: The summary for RR of clinical remission in IBD patients for four included trials comparing herbal medicines to placebo[16,17,19,21] was 2.07 with 95%CI: 1.41-3.03 ($P = 0.0002$, Figure 2A). The Cochrane Q test for heterogeneity indicated that the studies are not heterogeneous ($P = 0.08$, Figure 2B) and could be combined, thus fixed effects for individual and summary of RR was applied. Regression of normalized effect *vs* precision for all included studies for clinical remission in IBD patients among herbal medicines *vs* placebo therapy was 2.02 (95%CI: 0.37-3.67, $P = 0.03$ and Kendall's tau = 1, $P = 0.08$ (Figure 2C).

The RR of clinical remission in patients with CD[19] was 27 with 95%CI: 3.23-260.81, a significant RR.

The summary for RR of clinical remission in UC patients for two included trials comparing herbal medicines to placebo[16,17] was 1.59 with 95%CI: 0.8-3.15 ($P = 0.18$, Figure 3A). The Cochrane Q test for heterogeneity indicated that the studies are not heterogeneous ($P = 0.28$, Figure 3B) and could be combined but because of few included studies random effects for individual and summary of RR was applied. Regression of normalized effect *vs* precision for all included studies for clinical remission in UC patients could not be calculated because of too few strata.

Based on plant type, RR of clinical remission was significant for *Artemisia absinthium* (27.00; 95%CI: 3.23-260.81) and *Boswellia serrata* (2.34; 95%CI: 1.02-6.07) and non-significant for *Aloe vera* and *Andrographis paniculata* (Table 3).

Clinical response: The summary for RR of clinical response in IBD patients for five included trials comparing herbal medicines to placebo[16-19,22] was 2.59 with 95%CI: 1.24-5.42 ($P = 0.01$, Figure 4A). The Cochrane Q test for heterogeneity indicated that the studies are heterogeneous ($P = 0.08$, Figure 4B) and could not be combined, thus the random effects for individual and summary of RR was applied. Regression of normalized effect *vs* precision for all included studies for clinical response in IBD patients was 2.33 (95%CI: 1.55-3.11, $P = 0.003$) and Kendall's tau = 0.8, $P = 0.08$ (Figure 4C).

The summary for RR of clinical response in CD patients for two included trials[18,19] was 9.61 with 95%CI:

Table 3 Results obtained from sub-analyses based on plant type

Plant	IBD type	Study	Patients reported AE		Clinical efficacy		Endoscopic efficacy		Histological efficacy		Recurrence relapse
			Any AE	Serious AE	Clinical remission	Clinical response	Endoscopic remission	Endoscopic response	Histological remission	Histological response	
Aloe vera	UC	16	0.70 (0.25-2.08)	-	4.20 (0.84-24.84)	3.27 (1.06-12.13)	1.48 (0.44-5.84)	1.69 (0.69-5.04)	0.64 (0.25-1.81)	0.86 (0.55-1.55)	-
Andrographis paniculata	UC	17	0.94 (0.75-1.20)	1.01 (0.22-4.65)	1.41 (0.92-2.23)	1.32 (0.98-1.84)	1.32 (0.93-1.93)	-	-	-	-
Artemisia absinthium	CD	18	-	1.00 (0.06-16.69)	-	9.61 (0.73-126.15), $P = 0.09$	-	-	-	-	-
	CD	19	-	-	27.00 (3.23-260.81)		-	-	-	-	-
Boswellia serrata	CD	20	0.82 (0.66-1.04), $P = 0.11$	0.95 (0.27-3.31), $P = 0.94$	-	-	-	-	-	-	0.95 (0.52-1.73)
	Collagenous colitis	21			2.34 (1.02-6.07)	-	-	-	-	-	-
Triticum aestivum	UC	22	-	-	-	2.18 (1.19-4.78)	-	-	-	-	-

Results are expressed as relative risk (95%CI). AE: Adverse event; CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel disease.

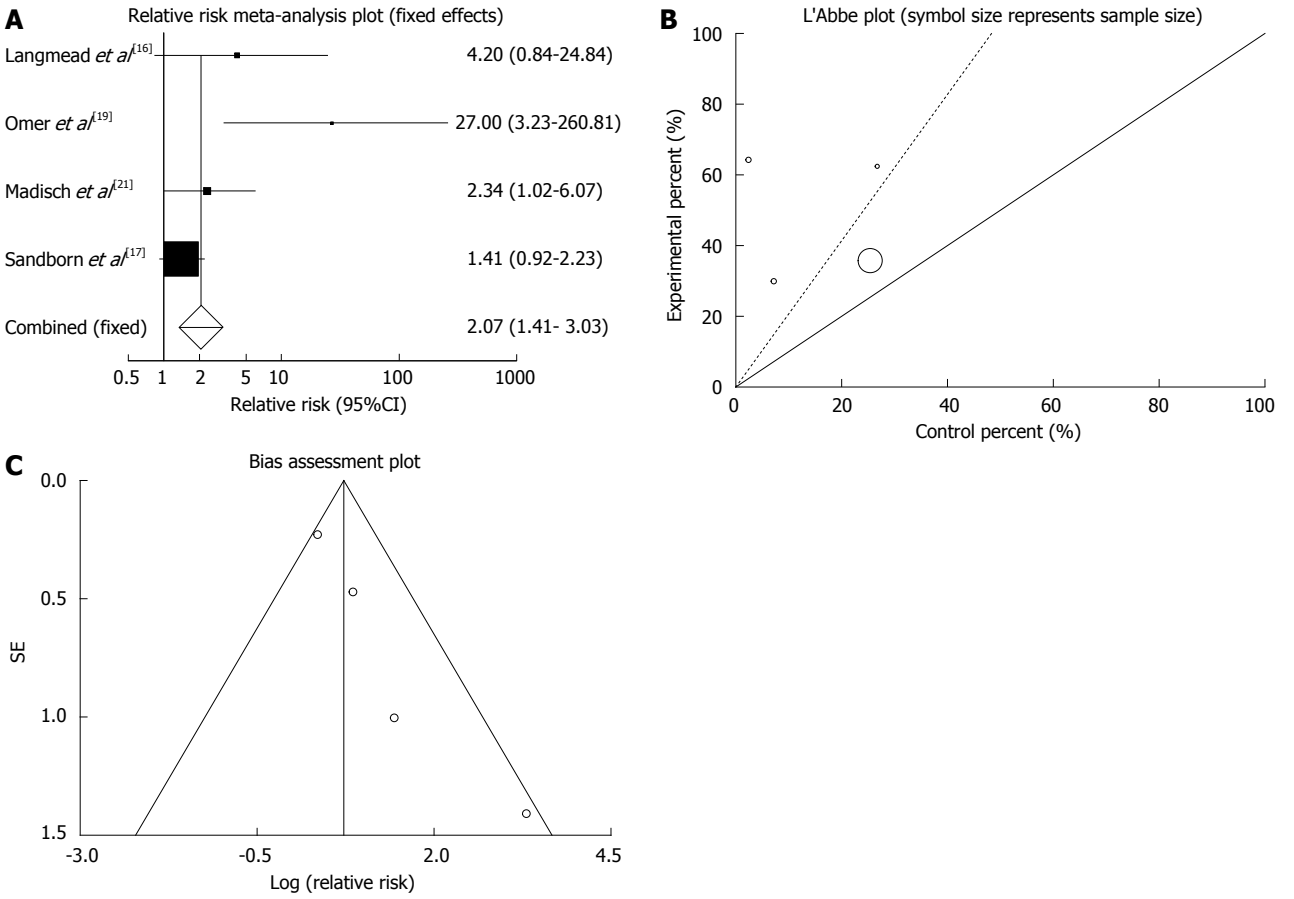

Figure 2 Individual and pooled relative risk (A), heterogeneity indicators (B) and publication bias indicators (C) for the outcome of "clinical remission" in the studies considering herbal medicines comparing to placebo therapy in inflammatory bowel disease patients.

0.73-126.15 ($P = 0.09$, Figure 5A). The Cochrane Q test for heterogeneity indicated that the studies are not heterogeneous ($P = 0.08$, Figure 5B) and could be combined but because of few included studies the random effects for individual and summary of RR was applied. Regression of normalized effect *vs* precision for all included studies for clinical response in CD patients could not be calculated because of too few strata.

The summary for RR of clinical response in UC patients for three included trials comparing herbal medicines to placebo[16,17,22] was 1.67 with 95%CI: 1.06-2.65 ($P = 0.03$, Figure 6A). The Cochrane Q test for heterogeneity indicated that the studies are not heterogeneous ($P = 0.22$, Figure 6B) and could be combined but because of few included studies the random effects for individual and summary of RR was applied. Regression of normalized effect *vs* precision for all included studies for clinical response in UC patients among herbal medicines *vs* pla-

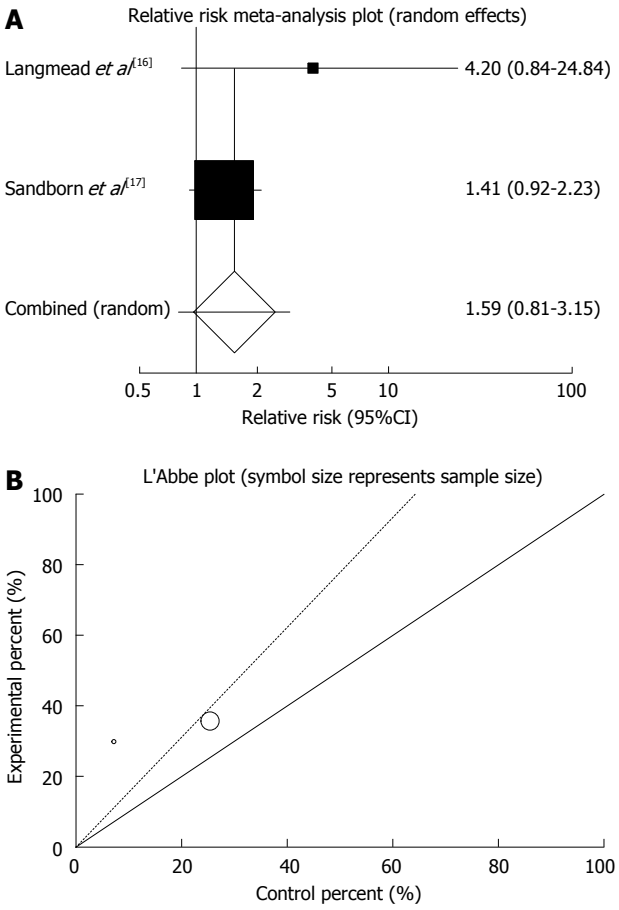

Figure 3 Individual and pooled relative risk (A) and heterogeneity indicators (B) for the outcome of "clinical remission" in the studies considering herbal medicines comparing to placebo therapy in ulcerative colitis patients.

cebo therapy could not be calculated because of too few strata.

Based on plant type, RR of clinical response was significant for *Aloe vera* (3.27; 95%CI: 1.06-12.13) and *Triticum aestivum* (2.18; 95%CI: 1.19-4.78) and non-significant for *Andrographis paniculata* and *Artemisia absinthium* (Table 3).

Endoscopic remission: The summary for RR of endoscopic remission in IBD patients for two included trials (all of the patients in these studies had UC) comparing herbal medicines to placebo[16,17] was 1.33 with 95%CI: 0.93-1.9 (P = 0.12, Figure 7A). The Cochrane Q test for heterogeneity indicated that the studies are not heterogeneous (P = 0.87, Figure 7B) and could be combined but because of few included studies random effects for individual and summary of RR was applied. Regression of normalized effect *vs* precision for all included studies for endoscopic remission in IBD (UC) patients could not be calculated because of too few strata.

Based on plant type, RR of endoscopic remission was non-significant for *Aloe vera* (1.48; 95%CI: 0.44-5.84) and *Andrographis paniculata* (1.32; 95%CI: 0.93-1.93) (Table 3).

Endoscopic response: The RR of endoscopic response in UC patients comparing herbal medicines with placebo[16] was 1.69 with 95%CI: 0.69-5.04, a non-significant RR.

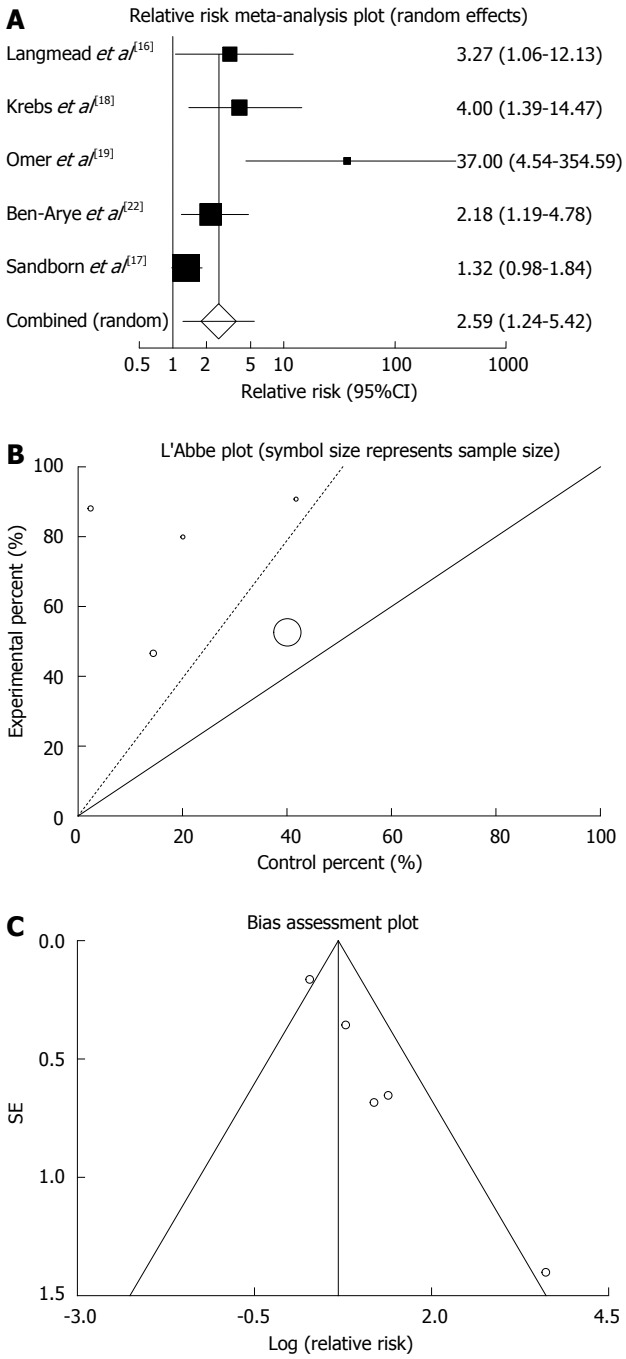

Figure 4 Individual and pooled relative risk (A), heterogeneity indicators (B) and publication bias indicators (C) for the outcome of "clinical response" in the studies considering herbal medicines comparing to placebo therapy in inflammatory bowel disease patients.

Histological remission: The RR of histological remission in IBD (UC) patients comparing herbal medicines with placebo[16] was 0.64 with 95%CI: 0.25-1.81, a non-significant RR.

Histological response: The RR of histological response in UC patients comparing herbal medicines with placebo[16] was 0.86 with 95%CI: 0.55-1.55, a non-significant RR.

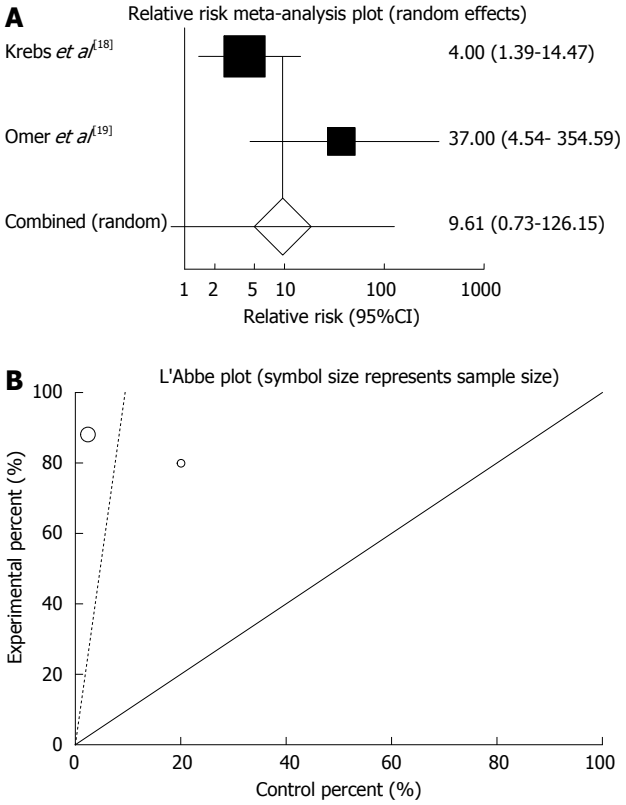

Figure 5 Individual and pooled relative risk (A) and heterogeneity indicators (B) for the outcome of "clinical response" in the studies considering herbal medicines comparing to placebo therapy in Crohn's disease patients.

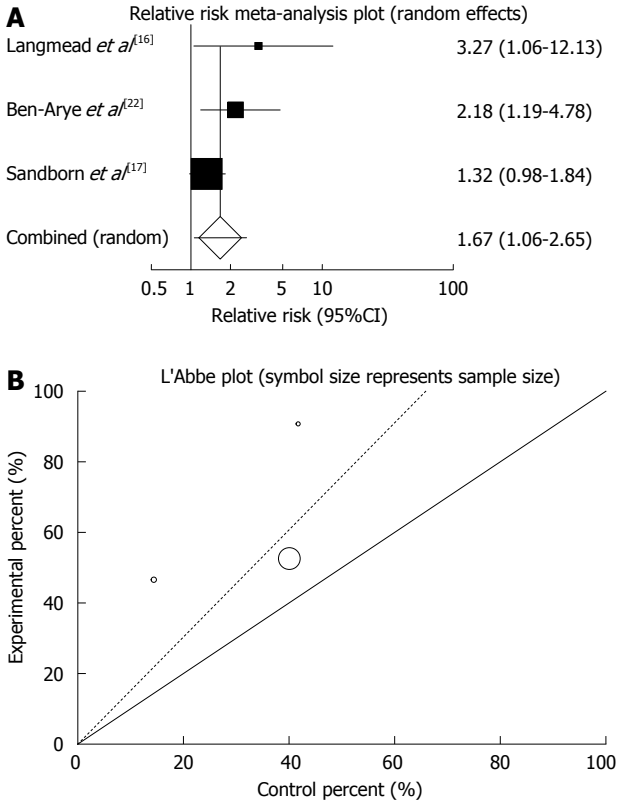

Figure 6 Individual and pooled relative risk (A) and heterogeneity indicators (B) for the outcome of "clinical response" in the studies considering herbal medicines comparing to placebo therapy in ulcerative colitis patients.

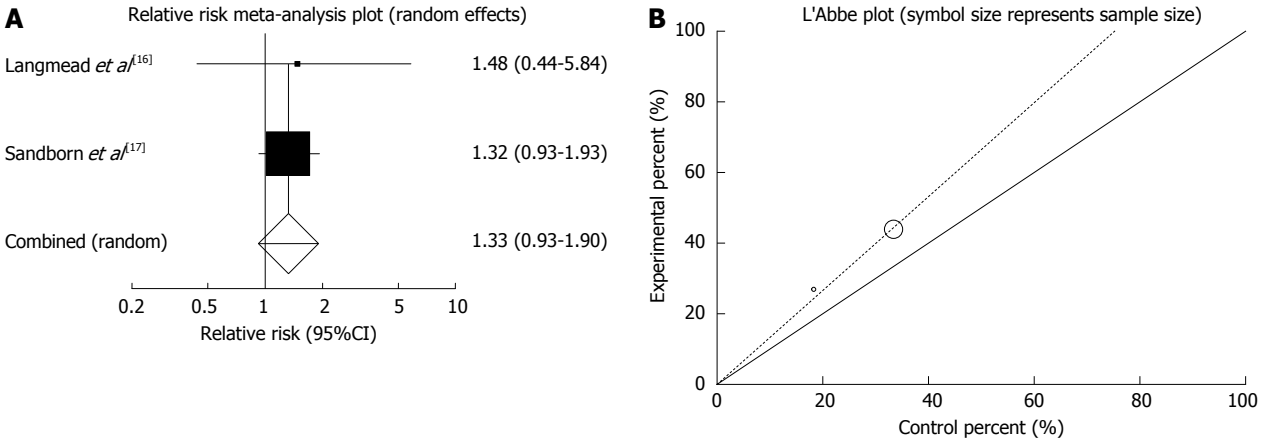

Figure 7 Individual and pooled relative risk (A) and heterogeneity indicators (B) for the outcome of "endoscopic remission" in the studies considering herbal medicines comparing to placebo therapy in inflammatory bowel disease (ulcerative colitis) patients.

Relapse: The RR of relapse in CD patients comparing herbal medicines with placebo[20] was 0.95 with 95%CI: 0.52-1.73, a non-significant RR.

Tolerability

Any adverse events: The summary for relative risk (RR) of any adverse events in IBD patients for four included trials comparing herbal medicines to placebo[16,17,20,21] was 0.89 with 95%CI: 0.75-1.06 ($P = 0.2$, Figure 8A). The Cochrane Q test for heterogeneity indicated that the studies are not heterogeneous ($P = 0.71$, Figure 8B) and could be combined, thus fixed effects for individual and summary of RR was applied. Regression of normalized effect *vs* precision for all included studies for any adverse events in IBD patients was 0.18 (95%CI: -2.73-3.09, $P = 0.81$) and Kendall's tau = 0, $P = 0.75$ (Figure 8C).

Serious adverse events: The summary for RR of serious adverse events in IBD patients for four included trials comparing herbal medicines to placebo[17,18,20,21] was

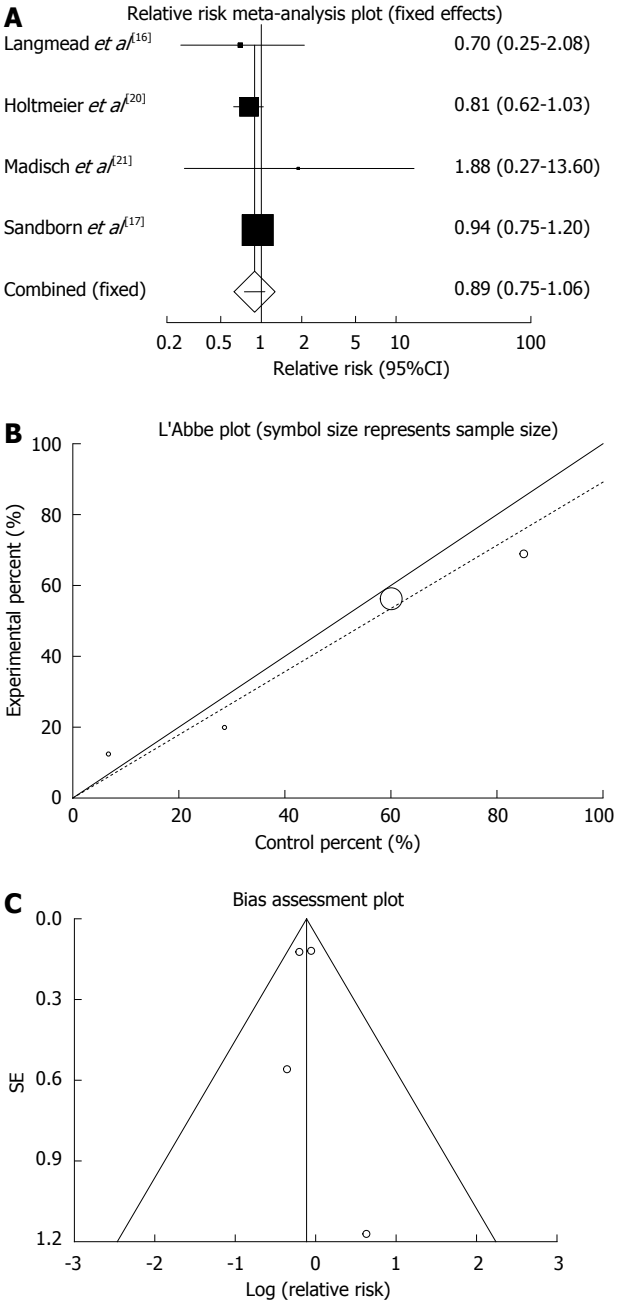

Figure 8 Individual and pooled relative risk (A), heterogeneity indicators (B) and publication bias indicators (C) for the outcome of "any adverse events" in the studies considering herbal medicines comparing to placebo therapy in inflammatory bowel disease patients.

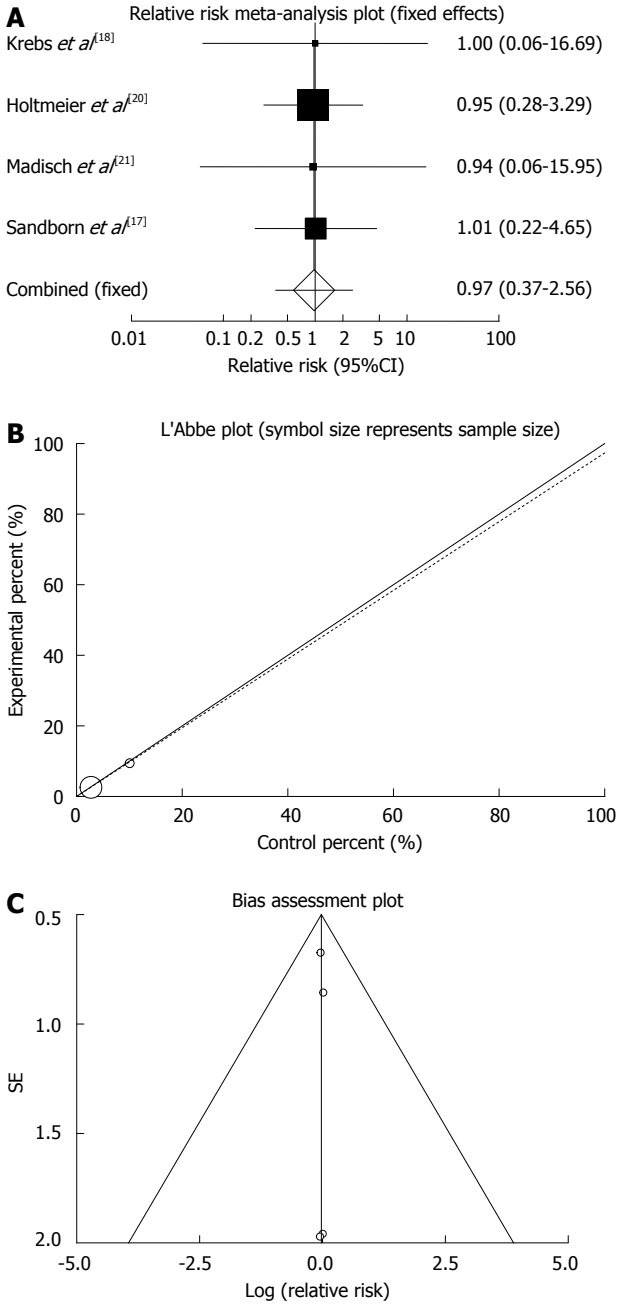

Figure 9 Individual and pooled relative risk (A), Heterogeneity indicators (B) and publication bias indicators (C) for the outcome of "serious adverse events" in the studies considering herbal medicines comparing to placebo therapy in inflammatory bowel disease patients.

0.97 with 95%CI: 0.37-2.56 (P = 0.96, Figure 9A). The Cochrane Q test for heterogeneity indicated that the studies are not heterogeneous (P > 0.99, Figure 9B) and could be combined, thus fixed effects for individual and summary of RR was applied. Regression of normalized effect *vs* precision for all included studies for serious adverse events in IBD patients was 0.01 (95%CI: -0.19-0.21, P = 0.83) and Kendall's tau = 0, P = 0.75 (Figure 9C).

DISCUSSION

In the current meta-analysis, the efficacy and tolerability of herbal medicines in the management all forms of IBD were compared with placebo. The results showed that herbal medicines may induce clinical remission and clinical response in patients with IBD. Endoscopic efficacy was investigated in two studies, both on patient with UC. Herbal medicines did not demonstrate significant effect on induction of endoscopic remission and endoscopic response. Histopathological efficacy was also evaluated in two studies both on patients with UC and the results were the same as endoscopic efficacy. This may be due to short duration of studies and possible slow action of herbal medicines. Moreover, the scoring system used to

assess the mucosal appearance macroscopically is prone to inter-observer variability resulting in non-detecting a significant improvement[23].

The efficacy of herbal medicines in prevention of relapse was investigated in only one study and showed no priority of these products compared to placebo. The number of patients showed any adverse events or serious adverse events were not significantly different between herbal medicines and placebo and this confirmed safety and tolerability of these products.

The present meta-analysis may have been limited by small sample sizes of studies and heterogeneity. Since the included trials involved herbal medicines contained different plants administered to patients with various subtypes of IBD, the trials were disaggregated. Thus, sub-analyses based on type of IBD and plant type was performed. The results of sub-analysis based on IBD type showed that herbal medicines significantly induce clinical remission in patients with CD and clinical response in patients with UC; however the induction of clinical remission in patients with UC and induction of clinical response in patients with CD by herbal medicines were not significant. The results of sub-analyses based on plant type demonstrated that induction of clinical remission was obtained only by *Artemisia absinthium* and *Boswellia serrata* and induction of clinical response was gained by only *Aloe vera* and *Triticum Aestivum*. None of the plants caused induction of endoscopic or histological efficacy. *Boswellia serrata* in one study evaluating recurrence rate did not cause prevention of relapse. Induction of adverse events by none of the plants was significant in comparison to that of placebo.

Overall, the results show that herbal medicines may induce clinical efficacy in patients with IBD, but the evidence is too limited to make any confident conclusions. Meta-analysis of clinical trials that have compared efficacy of herbal medicines with that of conventional drugs such as amino-salicylates can be helpful that is being carried out by authors of this paper. Further high quality, large controlled trials using standardized preparation are warranted to better elucidate the effects of these herbs in IBD.

ACKNOWLEDGMENTS

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COMMENTS

Background

Inflammatory bowel disease (IBD) is a group of inflammatory conditions of gastrointestinal tract. Due to lack of desired efficacy and poor tolerability of conventional drugs, approach toward complementary and alternative medicines especially herbal medicines for the management of IBD are growing. Besides many experimental studies, the efficacy and tolerability of herbal medicines in IBD have been investigated through several clinical trials.

Research frontiers

Although the efficacy and tolerability of herbal medicines for the management of IBD were evaluated through several clinical trials in comparison to placebo, no meta-analysis has been conducted to reach a convincing conclusion.

Innovations and breakthroughs

Based on this meta-analysis, herbal medicines may safely induce clinical response and remission in patients with IBD without significant effects on endoscopic and histological outcomes, but the number of studies is yet limited to make a strong conclusion.

Applications

Regarding desirable effects of herbal medicine in induction of clinical response and remission in IBD and their low adverse events, it would not be surprising to introduce good medicines to clinic if proper standardization and dose adjustments are done.

Peer review

The aim of the study was to evaluate the efficacy and tolerability of herbal medicines in IBD by conducting a meta-analysis. This paper is good for IBD community.

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CASE REPORT

Pancreatic paracoccidioidomycosis simulating malignant neoplasia: Case report

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Author contributions: Lima TB conceived and coordinated the study, and participated in data collection, acquisition of radiological figures, and manuscript writing; Domingues MAC participated in the study design, data collection, production of histopathological figures, and manuscript writing; Caramori CA, Silva GF, Oliveira CV, Yamashiro FS and Franzoni LC participated in data collection and manuscript writing; Sassaki LY and Romeiro FG coordinated the study and participated in data collection and manuscript writing.

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Abstract

Paracoccidioidomycosis is a systemic granulomatous disease caused by fungus, and must be considered in the differential diagnosis of intra-abdominal tumors in endemic areas. We report a rare case of paracoccidioidomycosis in the pancreas. A 45-year-old man was referred to our institution with a 2-mo history of epigastric abdominal pain that was not diet-related, with night sweating, inappetence, weight loss, jaundice, pruritus, choluria, and acholic feces, without signs of sepsis or palpable tumors. Abdominal ultrasonography (US) showed a solid mass of approximately 7 cm × 5.5 cm on the pancreas head. Abdominal computerized tomography showed dilation of the biliary tract, an enlarged pancreas (up to 4.5 in the head region), with dilation of the major pancreatic duct. The patient underwent exploratory laparotomy, and the surgical description consisted of a tumor, measuring 7 to 8 cm with a poorly-defined margin, adhering to posterior planes and mesenteric vessels, showing an enlarged bile duct. External drainage of the biliary tract, Roux-en-Y gastroenteroanastomosis, lymph node excision, and biopsies were performed, but malignant neoplasia was not found. Microscopic analysis showed chronic pancreatitis and a granulomatous chronic inflammatory process in the choledochal lymph node. Acid-alcohol resistant bacillus and fungus screening were negative. Fine-needle aspiration of the pancreas was performed under US guidance. The smear was compatible with infection by *Paracoccidioides brasiliensis*. We report a rare case of paracoccidioidomycosis simulating a malignant neoplasia in the pancreas head.

Key words: Paracoccidioidomycosis; Pancreas; Fungus infection; Pancreatic tumors; Differential diagnosis

Core tip: This is a report of a rare case of pancreatic paracoccidioidomycosis, which shows its importance in the differential diagnosis of intra-abdominal tumors in endemic areas. This is apparently the first such report written in English. The patient had a pancreatic mass adhering to vessels and deep planes, with enlargement of satellite lymph nodes; but malignant neoplasia was not found. The ultrasonography-guided pancreas fine-needle aspiration defined the diagnosis and successfully directed the therapy. Remarkably, although the patient had abdominal lymph node enlargement, he did not present peripheral lymphadenopathy, which is usually

the major complaint in patients with the juvenile form of paracoccidioidomycosis.

Lima TB, Domingues MAC, Caramori CA, Silva GF, Oliveira CV, Yamashiro FS, Franzoni LC, Sassaki LY, Romeiro FG. Pancreatic paracoccidioidomycosis simulating malignant neoplasia: Case report. *World J Gastroenterol* 2013; 19(34): 5750-5753 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i34/5750.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i34.5750

INTRODUCTION

Paracoccidioidomycosis (also known as Pb mycosis or South American blastomycosis) is a systemic granulomatous disease caused by the fungus *Paracoccidioides brasiliensis*[1], which is autochthonous in Latin America. It was described by Lutz[2], and its incidence ranges from 3 to 4 cases per million, up to 1 to 3 cases per one hundred thousand inhabitants per year in endemic areas, with an annual mortality of 1.45 per million inhabitants, which is the highest rate observed among systemic mycoses[1]. It affects 10 to 15 adult males per one female case, mostly between the 3rd and 6th decades of life, and has two main forms: the juvenile form, constituting 3%-5% of cases, which compromises the reticuloendothelial system, and the somewhat more common chronic form that comprises 90% of cases, mainly affecting the skin and the lungs[1]. Paracoccidioidomycosis can mimic neoplasias, such as periampullary[3] or colon[4] cancer. Therefore, it must be considered in the differential diagnosis of intra-abdominal tumors in endemic areas[5]. This case illustrates a rare case of paracoccidioidomycosis simulating a malignant neoplasia in the pancreas head.

CASE REPORT

A 45-year-old white adult male from São Paulo state was referred to our institution with a 2-mo history of epigastric pain that was not diet-related. It was intermittent, of strong intensity, with radiation to the back and relieved by the use of omeprazole. Additionally, the patient had night sweating and a weight loss of 13 kg in 45 d, and lacked vomiting, diarrhea, or fever. Twenty days before admission, the patient reported jaundice, pruritus over the whole body, choluria, and acholic feces. The patient was a current smoker and alcohol user, while his physical examination revealed jaundice and pain after deep abdominal palpation, without signs of sepsis or palpable tumors. Pulmonary auscultation showed no alterations, and laboratory exams confirmed cholestatic syndrome (Table 1).

Abdominal ultrasonography (US) showed a solid mass of approximately 7 cm × 5.5 cm on the pancreas head, with a small tumor of 1 to 2 cm juxtaposed with the mass, suggesting local metastases. Abdominal computerized tomography (CT) showed dilation of the biliary tract, an enlarged pancreas (up to 4.5 in the region of the head) with a poorly-defined margin and two hypodense areas without significant enhancement after intravenous contrast, leading to dilation of the major pancreatic duct, but without retroperitoneal lymphadenopathy (Figure 1). Due to the momentary unavailability of the necessary equipment at our institution to perform a puncture guided by CT or US, the patient underwent exploratory laparotomy. The surgical description consisted of a pancreatic tumor measuring 7 to 8 cm with a poorly-defined margin, adhering to posterior planes and to mesenteric vessels, and showing an enlarged bile duct. Transcystic cholangiography showed obstruction of the passage of contrast through the distal bile duct, which was compatible with extrinsic compression. External drainage of the biliary tract, Roux-en-Y gastroenteroanastomosis, lymph node excision, and biopsies were performed. Malignant

Table 1 Laboratory exams according to the time of hospitalization

	Admission	3 mo	12 mo	Normal range
GGT (U/L)	646	677	632	15-73
Alkaline phosphatase (U/L)	1047	600	262	36-126
Aspartate transaminase (U/L)	58	147	68	30-110
Alanine transferase (U/L)	53	184	173	21-75
Albumin (g/dL)	2.5	4.3	4.8	3.5-5
INR	1.04	1.17	1.12	< 1.25
Total bilirubin (mg/dL)	3.8	1.7	0.6	0.2-1.3
Indirect bilirubin (mg/dL)	0.6	0.4	0.2	0-1.1
Direct bilirubin (mg/dL)	1.2	0.1	0	0-0.3
Platelets (× 10^3/mm^3)	278	177	138	140-440
Leukocytes (× 10^3/mm^3)	6.2	4.5	5.3	4-11

GGT: Gamma-glutamyl transpeptidase; INR: International normalized ratio.

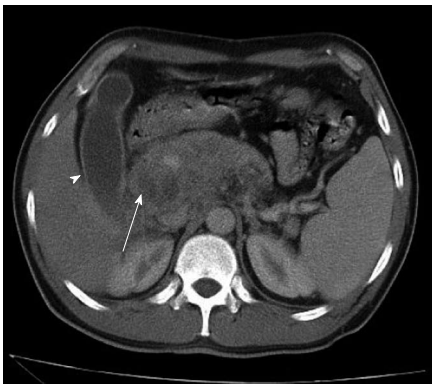

Figure 1 Contrast enhanced abdominal computed tomography. Head, body, and tail of pancreas with enlarged dimensions and a poorly-defined margin, with two hypodense areas without significant enhancement after intravenous contrast (white arrow). The same image shows a hydropic gallbladder (arrow head) and mild splenomegaly.

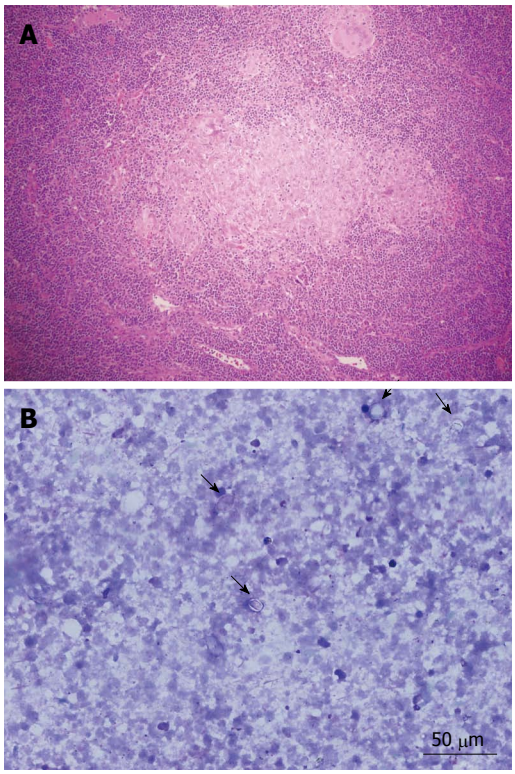

Figure 2 Pathological findings (hematoxylin/eosin × 200 magnification). A: Epithelioid granuloma in a lymph node near the ductus choledochus, with absence of necrosis, fungi, or bacteria; B: Fine-needle aspiration smear showing rounded yeast forms with a birefringent wall and multiple sporulation compatible with *Paracoccidioides brasiliensis* (black arrows).

neoplasia was not found at the intraoperative frozen section analysis. Microscopic analysis showed chronic pancreatitis and a granulomatous chronic inflammatory process in the choledochal lymph node. The presence of granulomas without caseous necrosis in the lymph node would suggest a diagnosis of sarcoidosis; however, at that time it was not possible to definitively discard the diagnosis of tuberculosis (Figure 2A). Acid-alcohol resistant bacillus and fungus screening by Ziehl-Neelsen, PAS, and Gömöri staining were negative. The biopsies were reviewed, and a cohesive granuloma without caseous necrosis was found attached to peripancreatic fat. The abdominal pain and the jaundice worsened, and the patient developed fever, leukocytosis, and drainage of purulent secretion by a surgical drain located near the pancreas. Upper digestive endoscopy showed esophageal varices. Plain chest X-ray and thoracic CT scan were normal. Metronidazole and ciprofloxacin were prescribed due to the hypothesis of bacterial cholangitis, and clinical improvement was observed. Fine-needle aspiration (FNA) of the pancreas was performed with US guidance. The obtained smear was compatible with infection by *Paracoccidioides brasiliensis* (Figure 2B). Enzyme-linked immunosorbent assay tests, employed for the detection of anti-*Paracoccidioides brasiliensis* antibodies, produced positive results (titers of 1/8 and 1/16). Intravenous sulfamethoxazole-trimethoprim (800/160 mg two times per day) was introduced, after which the patient showed clinical and laboratory improvement (Table 1).

DISCUSSION

Pancreatic paracoccidioidomycosis simulating pancreatic neoplasia has been infrequently reported in the literature[6]. Clinical findings include weight loss, weakness, dizziness, repletion, pruritus, jaundice, choluria, and fecal acholia, as in the reported case. Signs of cholestatic disease are the most reported abnormalities, with an increase in alkaline phosphatase and gamma-glutamyl transferase. Jaundice is usually observed in the late stage of the disease, and is associated with differential diagnoses of greater severity[7], such as malignant pancreatic neoplasia. The findings of pancreatic tumor adhering to vessels and deep planes, as well as satellite nodules and obstruction of the biliary tract with secondary portal hypertension (diagnosed through the esophageal varices), suggested a diagnosis of metastatic pancreatic cancer. As shown in this report, abdominal lymphatic compromise may give rise to such clinical conditions as abdominal tension and pain, which may even simulate acute abdomen affections[8]. The granulomatous involvement of lymph nodes initially led to the hypothesis of sarcoidosis, which may affect multiple organs, particularly the lungs (90%) and lymph nodes (75%)[9]. But sarcoidosis rarely affects the pancreas[10,11], and when it does, it is usually asymptomatic[12]. The type of granuloma found in the lymph node, characteristically epithelioid, without necrosis or bacilli, favored this diagnosis. On the other hand, immunosuppression with corticoids, which would be recommended in a case of sarcoidosis, may be harmful if the patient has had an infectious disease. At that time, the second pancreatic tissue sample obtained by FNA defined the paracoccidioidomycosis diagnosis and successfully directed the therapy. Interestingly, although the patient had had an abdominal lymph node enlargement, he did not have peripheral lymphadenopathy, which is usually the major complaint in patients with the juvenile form of paracoccidioidomycosis. Additionally, there were none of the pulmonary manifestations that occur in 90% of patients with the chronic form[1]. Although it is rare, pancreatic paracoccidioidomycosis must be considered in the differential diagnosis of intra-abdominal tumors in endemic areas, even without peripheral lymphadenopathy.

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CASE REPORT

Extended therapy duration for therapy-refractory hepatitis C patients with genotype 2

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Abstract

We devised an extended 72-wk peginterferon-α-2a/ribavirin therapy regimen for the retreatment of highly intractable cases, *i.e.*, 48-wk peginterferon-α-2b/ribavirin therapy-intractable cases. Although 2 cases achieved a rapid virological response to 72-wk peginterferon-α-2a/ribavirin therapy, 1 case failed to achieve a sustained virological response. Although the reason for this difference in the effectiveness of 72-wk peginterferon-α-2a/ribavirin therapy between the cases was unclear, the rebound phenomenon of serum transaminase after 48-wk peginterferon-α-2b/ribavirin therapy and the resultant lower viral load compared to that before 48-wk peginterferon-α-2b/ribavirin therapy might have influenced the treatment outcome. Thus, it may be beneficial to consider the rebound phenomenon of serum transaminase and the changes in viral load resulting from previous interferon-based therapy and then cautiously determine the indication and the timing of the administration of 72-wk peginterferon-α-2a/ribavirin in highly intractable cases. Further studies should be performed to confirm this strategy.

Key words: Hepatitis C; Genotype 2 and high viral loads; Interferon-based therapy; Highly intractable case; Extended therapy duration

Core tip: The optimal therapy for 48-wk peginterferon-α-2b/ribavirin therapy-intractable hepatitis C patients with genotype 2 and high viral loads remains unknown. Our cases are notable in that 72-wk peginterferon-α-2a/ribavirin therapy may have been effective for these highly intractable cases. Additionally, the rebound phenomenon of serum transaminase after the 48-wk peginterferon-α-2b/ribavirin therapy and the resultant lower viral load compared to that before the 48-wk peginterferon-α-2b/ribavirin therapy might have influenced the treatment outcome. Thus, our cases highlight the importance of the results of the previous 48-wk peginterferon-α-2b/ribavirin therapy in the indication and timing of the administration of 72-wk peginterferon-α-2a/ribavirin in highly intractable cases.

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INTRODUCTION

The American Association for the Study of Liver Diseases Practice Guidelines recommend treatment with

peginterferon/ribavirin for 24 wk using a ribavirin dose of 800 mg for interferon-based therapy-naïve patients with hepatitis C virus (HCV) genotype 2[1]. However, retreatment with peginterferon/ribavirin in patients who do not achieve a sustained virological response (SVR) after a full course of peginterferon/ribavirin is not recommended, even if a different type of peginterferon is administered[1]. However, some patients who have previously completed peginterferon-α-2b [PegIntron; Merck Sharp & Dohme (MSD), Tokyo, Japan]/ribavirin (Rebetol; MSD) therapy but failed to achieve an SVR can be treated with a 24- to 48-wk course of peginterferon/ribavirin[2-4]. Oze *et al*[2] suggested that the SVR rate was similar between 24-wk and 48-wk peginterferon/ribavirin therapy in patients with genotype 2 who achieved a rapid virological response (RVR) upon retreatment. We previously demonstrated the superiority of 48-wk therapy over 24-wk therapy for patients with genotype 2 and high viral loads $\geqslant 10^5$ international units (IU)/mL; 5 logIU/mL as determined using the quantitative reverse transcription polymerase chain reaction-based Cobas TaqMan HCV Test (Roche Diagnostics, Tokyo, Japan) who demonstrated serum HCV RNA levels [measured using the Cobas Amplicor Monitor HCV ver. 2.0 Assay (Roche Diagnostics)] $\geqslant$ 50 IU/mL at week 4 of therapy regardless of the history of interferon-based therapy[5]. The Japan Society of Hepatology also recommends a 24- to 48-wk course of peginterferon/ribavirin retreatment following peginterferon/ribavirin treatment in patients with HCV genotype 2 and high viral loads[6]. However, the effectiveness of retreatment in highly intractable cases, *i.e.*, 48-wk peginterferon-α-2b/ribavirin therapy-intractable cases, remains unknown. Therefore, we devised a 72-wk peginterferon/ribavirin therapy regimen for the retreatment of highly intractable cases, regardless of the time of disappearance of serum HCV RNA.

As for the type of peginterferon, the superiority of peginterferon-α-2a therapy over peginterferon-α-2b therapy for chronic hepatitis C has not been determined. However, given that peginterferon-α-2a therapy is significantly superior or has a tendency to increase the efficacy of treatment in patients with HCV genotype 2 compared to peginterferon-α-2b therapy according to randomized controlled trials[7,8] and a meta-analysis of randomized controlled trials[9], we selected peginterferon-α-2a (Pegasys; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan)/ribavirin (Copegus; Chugai Pharmaceutical Co., Ltd.) for the retreatment of 48-wk peginterferon α-2b/ribavirin therapy-intractable cases.

The submitted case reports comply with the Declaration of Helsinki. Informed consent was obtained from the patients.

CASE REPORT

Case 1

A 55-year-old male was referred to our hospital because of chronic HCV infection and abnormal transaminase levels. His medical history included an operation for the treatment of appendicitis at 8 years of age and acute hepatitis with jaundice at 23 years of age after he had acquired a tattoo and abused drugs at 25 years of age. He had neither a history of blood transfusion nor a family history of liver diseases. The transmission of hepatitis C in this case could have been mediated by either the tattoo or drug abuse. His complete blood counts appeared normal, although blood tests showed elevated levels of serum alanine aminotransferase (ALT) (44 IU/mL). The quantitative detection of HCV RNA [real-time polymerase chain reaction (PCR), COBAS TaqMan Test] revealed a level of 6.5 logIU/mL; the HCV genotype was 2b. The interleukin-28B (IL28B) locus single nucleotide polymorphisms (SNPs) previously reported to be associated with therapy outcome, including rs8099917, rs11881222 and rs8103142[10], were all major homozygous. The inosine triphosphatase (ITPA) locus SNP previously reported to be associated with ribavirin-induced hemolytic anemia[11] and interferon-induced thrombocytopenia[12] in Japanese chronic hepatitis C patients under peginterferon-α/ribavirin therapy (rs1127354) was major homozygous. A liver biopsy obtained prior to interferon-based therapy was graded F1/A2 according to the New Inuyama classification.

We initiated treatment with peginterferon-α-2b (100 μg once weekly, subcutaneously) and ribavirin (800 mg per day) based on the patient's body weight. Serum HCV RNA was not detectable at wk 8 of therapy. Thus, we extended the duration of therapy from the standard 24-wk regimen to a 48-wk regimen based on our prospective study[5]. The patient's adherence to peginterferon-α-2b/ribavirin was 100%, although his serum HCV RNA level became positive 4 wk after the completion of therapy. Notably, the rebound phenomenon of serum transaminase after completion of the 48-wk therapy occurred, and the viral load decreased below the pre-treatment level. He was retreated with peginterferon-α-2a (180 μg once weekly, subcutaneously) and ribavirin (800 mg per day) based on his body weight. Because the 48-wk therapy with peginterferon-α-2b and ribavirin resulted in viral relapse, we extended the duration of therapy from the recommended 24-48 wk to 72 wk, although the serum level of HCV RNA became undetectable after 4 wk of therapy. Although the patient's adherence to ribavirin was 74% due to anemia, he achieved an SVR. The laboratory findings, treatments and outcomes for case 1 are shown in Table 1.

Case 2

A 55-year-old male was referred to our hospital because of chronic HCV infection. His previous medical history included an operation due to appendicitis at 14 years of age and euthyroid sick syndrome, the onset of which was unclear. He had neither a history of blood transfusion nor a family history of liver disease. Thus, the transmission source of hepatitis C in this case could not be

Table 1 Laboratory findings at baseline, treatments and outcomes of highly intractable cases

Parameters	Case 1		Case 2	
Age (yr)	55		55	
Sex	Male		Male	
HCV genotype	2b		2b	
Transmission	Tattoo or drug abuse		Unknown	
IL28B SNPs (rs8099917:rs11881222:rs8103142)	T/T:A/A:T/T		T/T:A/A:T/T	
ITPA SNPs	C/C		C/C	
At the start of therapy	First therapy	Second therapy	First therapy	Second therapy
BMI (kg/m^2)	26	27.5	20.9	19.7
HCV RNA (logIU/mL)	6.5	3.7	7.2	7.5
Liver biopsy	F1/A2	Not performed	F1/A2	Not performed
ALT (IU/L)	36	26	32	40
AST (IU/L)	44	32	32	33
WBC (cells/μL)	4520	3880	3690	4020
Neutrophil (cells/μL)	2650	2420	1450	1950
Hemoglobin (g/dL)	15.7	14.6	11.9	12.9
Platelets (cells/μL)	16.4	15.7	14.5	15.3
Peak ALT after the first therapy (IU/L)	210		33	
Duration between the first therapy and the second therapy (wk)	17		10	
Treatment and outcome	First therapy	Second therapy	First therapy	Second therapy
Peginterferon α dosage (μg)	100	180	100	180
RBV dosage (mg)	800	800	800	800
Week at disappearance of serum HCV RNA	8	4	16	4
Adherence to peginterferon α	100%	100%	100%	100%
Adherence to RBV	100%	74%	96%	100%
Weeks of therapy	48	72	48	72
Response	Relapse	SVR	Relapse	Relapse

HCV: Hepatitis C virus; IL28B: Interleukin-28B; SNPs: Single nucleotide polymorphisms; ITPA: Inosine triphosphatase; first therapy: 48-wk peginterferon α-2b/ribavirin; second therapy: 72-wk peginterferon α-2a/ribavirin; BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; WBC: White blood cell; RBV: Ribavirin; SVR: Sustained virological response.

identified. A complete blood count showed slight anemia, and blood tests showed a slightly high serum ALT level (32 IU/mL). The quantitative detection of HCV RNA (real-time PCR, COBAS TaqMan Test) revealed a level of 7.2 logIU/mL; the HCV genotype was 2b. The IL28B locus SNPs, including rs8099917, rs11881222 and rs8103142, were all major homozygous. The ITPA locus SNP rs1127354 was major homozygous. A liver biopsy obtained prior to interferon-based therapy was graded F1/A2 according to the New Inuyama classification.

We initiated treatment with peginterferon-α-2b (100 μg once weekly, subcutaneously) and ribavirin (800 mg per day) based on the patient's body weight. Serum HCV RNA disappeared at week 16 of therapy. Thus, we extended the therapy duration from the standard 24-wk regimen to a 48-wk regimen based on our prospective study[5]. Although the patient's adherence to peginterferon-α-2b/ribavirin was 100%, his serum HCV RNA level became positive 4 wk after the completion of therapy. In contrast to case 1, no obvious rebound phenomenon of serum transaminase was observed after completion of the 48-wk therapy, and the viral load returned to the pre-treatment level. He was retreated with peginterferon-α-2a (180 μg once weekly, subcutaneously) and ribavirin (800 mg per day) based on his body weight. Because the 48-wk therapy with peginterferon-α-2b and ribavirin resulted in viral relapse, we extended the duration of therapy from the recommended 24-48 wk to 72 wk, and his serum HCV RNA level became negative at wk 4 of therapy. Although the patient's adherence to both drugs was 100%, his serum HCV RNA level became positive again at 4 wk after the completion of therapy, and he therefore could not achieve an SVR. The laboratory findings, treatments and outcomes of case 2 are shown in Table 1.

DISCUSSION

The major findings from these case reports are twofold: 72-wk peginterferon-α-2a/ribavirin therapy may represent an effective therapy for 48-wk peginterferon-α-2b/ribavirin therapy-intractable cases, and by contrast, even 72-wk peginterferon-α-2a/ribavirin therapy-intractable HCV infection can occur in patients with genotype 2 and high viral loads. Although the difference in the effectiveness of 72-wk therapy between the 2 cases was not clear, the rebound phenomenon of serum transaminase after the 48-wk therapy and the resultant lower viral load compared to that before the 48-wk therapy might be possible contributing factors that could influence the treatment outcome, based on these case reports and our previous study[5]. Case 1 was consistent with a previous

report indicating that patients who showed biochemical relapse after initial interferon therapy had a significantly lower serum HCV RNA level at recovery after ALT relapse compared to before the initial interferon therapy[13]. Moreover, our cases showed that an RVR was not sufficient for predicting an SVR, even for 72-wk interferon-based therapy. Because both of our cases carried major homozygous IL28B SNPs, our findings support a limited role for *IL28B* genotypes regarding the virological responses achieved in chronic hepatitis C patients with genotype 2 and high viral loads[5]. However, because the number of our highly intractable cases was small, further studies are needed to test this hypothesis.

One possible solution for the treatment of highly intractable cases is telaprevir in combination with peginterferon-α-2b/ribavirin therapy. In fact, this therapy achieved an SVR in a 48-wk peginterferon-α-2b/ribavirin therapy-intractable female patient with genotype 2 and a high viral load in a phase Ⅲ clinical trial (unpublished data). In addition, direct-acting anti-viral (DAA) combination therapy may overcome this difficulty in highly intractable cases in the near future. However, for cases in which it is difficult to use DAA due to the risk of drug interactions between DAA and medicines administered to treat complications or for patients who are discouraged from waiting for antiviral therapy, such as those who developed hepatocellular carcinoma, 72-wk peginterferon-α-2a/ribavirin therapy may be one strategy for curing highly intractable patients with genotype 2 and high viral loads. For case 1 presented here, 24- to 48-wk peginterferon-α-2a/ribavirin therapy for retreatment may have been effective, although we selected 72-wk therapy to improve the likelihood of an SVR. However, 72-wk therapy is not always sufficient, as demonstrated in case 2 (although he did achieve an RVR). In case 1, the viral load after 48-wk peginterferon-α-2b/ribavirin therapy decreased significantly in comparison to the level prior to treatment, whereas the viral load returned to the pre-treatment level in case 2. We were able to initiate the 72-wk therapy under the condition that the viral load had become lower than that before the 48-wk therapy in case 1. This indicates that it may be a good strategy to consider the rebound phenomenon of serum transaminase and the changes in viral load as a result of previous interferon-based therapy and then cautiously determine the indication and timing of administration of the 72-wk peginterferon-α-2a/ribavirin in highly intractable cases. However, a therapy duration of less than 72 wk should also be considered as a second therapy if the pre-treatment viral load before the second therapy is low (less than 5 logIU/mL) because chronic hepatitis C patients with low viral loads are likely to achieve an SVR in short-term therapy. Further studies should be examined to confirm the strategy of extended therapy duration.

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CASE REPORT

Cerebral and splenic infarctions after injection of *N*-butyl-2-cyanoacrylate in esophageal variceal bleeding

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Author contributions: Myung DS and Joo YE designed the report; Chung CY, Park HC and Kim JS organized the report; Cho SB was the physician for this patient, discussed the treatment plan with the patient, and revised the manuscript; Lee WS and Choi SK provided intellectual advice and revised the manuscript; Myung DS and Joo YE wrote the paper.

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Abstract

Variceal bleeding is the most serious complication of portal hypertension, and it accounts for approximately one fifth to one third of all deaths in liver cirrhosis patients. Currently, endoscopic treatment remains the predominant method for the prevention and treatment of variceal bleeding. Endoscopic treatments include band ligation and injection sclerotherapy. Injection sclerotherapy with *N*-butyl-2-cyanoacrylate has been successfully used to treat variceal bleeding. Although injection sclerotherapy with *N*-butyl-2-cyanoacrylate provides effective treatment for variceal bleeding, injection of *N*-butyl-2-cyanoacrylate is associated with a variety of complications, including systemic embolization. Herein, we report a case of cerebral and splenic infarctions after the injection of *N*-butyl-2-cyanoacrylate to treat esophageal variceal bleeding.

Key words: Cerebrum; Esophageal varix; Infarction; *N*-butyl-2-cyanoacrylate; Spleen

Core tip: Variceal bleeding is the most serious complication of portal hypertension, and it accounts for approximately one fifth to one third of all deaths in liver cirrhosis patients. Although injection sclerotherapy with *N*-butyl-2-cyanoacrylate provides effective treatment for variceal bleeding, injection of *N*-butyl-2-cyanoacrylate is associated with a variety of complications including systemic embolization. Herein, we report a case of cerebral and splenic infarctions after the injection of *N*-butyl-2-cyanoacrylate to treat esophageal variceal bleeding.

Myung DS, Chung CY, Park HC, Kim JS, Cho SB, Lee WS, Choi SK, Joo YE. Cerebral and splenic infarctions after injection of *N*-butyl-2-cyanoacrylate in esophageal variceal bleeding. *World J Gastroenterol* 2013; 19(34): 5759-5762 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i34/5759.htm
DOI: http://dx.doi.org/10.3748/wjg.v19.i34.5759

INTRODUCTION

Variceal bleeding is a catastrophic complication of liver cirrhosis. Although the short-term mortality of variceal bleeding has improved due to recent advances in treatment, the long-term outcomes remain guarded.

Currently, endoscopic treatment remains the predominant method for prevention and treatment of variceal bleeding. Endoscopic treatments include band ligation and injection sclerotherapy. Injection sclerotherapy with *N*-butyl-2-cyanoacrylate (Histoacryl, B-Braun Surgical GmbH, Melsungen, Germany) has been successfully used for variceal bleeding, but Histoacryl injection is associated with a variety of complications, some of which can be disastrous[1].

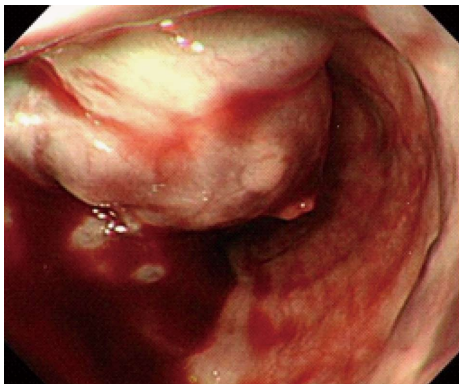

Figure 1 Endoscopy showing a large esophageal varix. The varix occupies more than half of the esophageal lumen. The adherent, whitish fibrin plug on top of the varix is considered a site of recent hemorrhage.

Systemic embolization including the cerebrum, lung, spleen, and portal vein is a rare and serious complication of Histoacryl injection that has been principally described in the treatment of gastric variceal bleeding[2,3]. However, this complication following the esophageal variceal injection of Histoacryl is extremely rare. To date, few cases of this complication at one site have been reported in the English literature.

To our knowledge, this is the first report of a case of multiple embolizations including the cerebrum and spleen after Histoacryl injection in esophageal variceal bleeding.

CASE REPORT

A 55-year-old woman with alcohol-induced liver cirrhosis of Child-Pugh class B was admitted to Chonnam National University Hwasun Hospital (Jeonnam, South Korea) with a 1-d history of hematemesis. She denied prior gastrointestinal bleeding, peptic ulcer diseases, and use of ulcerogenic medications. On admission, she had a pulse of 90 beats/min, a blood pressure of 80/50 mmHg, and a respiratory rate of 30 breaths/min. The head and neck examination was normal, except for anemic conjunctiva. She had florid spider angiomata. The abdomen was nontender with ascites, and the spleen tip was slightly palpable. Rectal examination demonstrated the presence of maroon-colored, liquid stool. Laboratory studies revealed the following: hemoglobin, 8.2 g/dL (normal range, 12-18 g/dL); hematocrit, 24.8% (37%-52%); white blood cell count, 9300/mm^3 (4000-10800/mm^3); platelet count, 100000/mm^3 (130000-450000/mm^3); total protein, 5.1 g/dL (5.8-8.1 g/dL); albumin, 2.3 g/dL (3.1-5.2 g/dL); total bilirubin, 1.4 mg/dL (0.3-1.3 mg/dL); aspartate aminotransferase, 90 U/L (7-38 U/L); and alanine aminotransferase, 8 U/L (6-42 U/L). Her coagulation profiles were prothrombin time 19.6 s (11-14.9 s) and activeated partial thromboplastin time 34.5 s (28-40 s). Endoscopy showed a large esophageal varix with a fibrin plug (Figure 1). Because the bleeding esophageal varix was too large to apply band ligation, we performed injection sclerotherapy

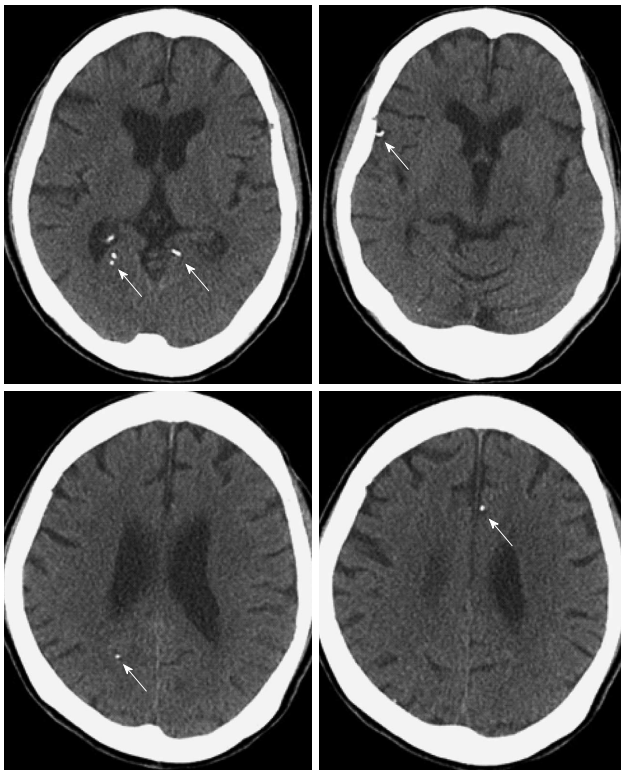

Figure 2 Non-contrast brain computed tomography showing multiple high attenuation lesions (arrows). The multiple high attenuation lesions are emboli of the Histoacryl-Lipiodol mixture. The high attenuation lesions were seen in the frontal lobe and the parieto-occipital lobe.

with a mixture of Histoacryl and Lipiodol (Laboratoire Guerbet, Aulnay-Sous-Bois, France). The mixture was injected intra-variceally using a 21-gauge needle injector (Injector Force, NM-200L-0821, Olympus Optical Co., Ltd., Tokyo, Japan). The mixture consisted of 0.5 mL of Histoacryl and 0.5 mL of Lipiodol. Because variceal bleeding was not controlled after the first injection, a second injection was performed in the same manner. After the second injection, variceal bleeding was controlled. The total injected volume was 2 mL. However, she developed dysarthria and right motor weakness (grade Ⅲ/Ⅴ) 1 h after the injection. Brain computed tomography (CT) showed multiple hyperdense foci in the frontal lobe and the parieto-occipital lobe (Figure 2). Magnetic resonance imaging of the brain showed acute multifocal cortical infarctions. Abdominal CT revealed several wedge-shaped, low attenuation lesions in the spleen, indicating infarction (Figure 3). To evaluate the cause of the newly developed cerebral and splenic infarctions, a transcranial Doppler (TCD) bubble test was performed. The TCD bubble test is used to detect a right-to-left shunt. We used 2 MHz M-mode TCD (ST3, Spencer Technologies, Seattle, Washington, United States; SONARA, Viasys Healthcare, Conshohocken, Pennsylvania, United States) to detect microbubbles in the middle cerebral artery. TCD demonstrated the presence of a microbubble on the M-mode displays in the middle cerebral artery. TCD using Spencer Logarithmic Scale Grades was indicative of grade Ⅲ during resting and grade Ⅳ during the Valsalva maneuver

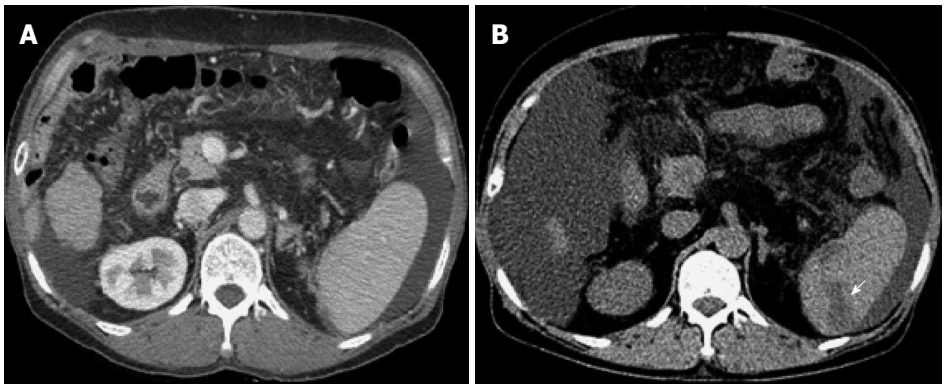

Figure 3 Abdominal computed tomography reveals several wedge-shaped, low attenuation lesions in the spleen, indicating infarction (arrow). A: Computed tomography (CT) before endoscopic treatment; B: CT after endoscopic treatment.

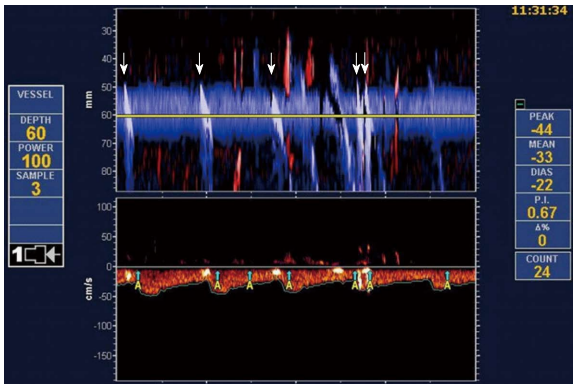

Figure 4 Transcranial Doppler bubble test showing the Doppler flow through the middle cerebral artery. The embolus is clearly represented in the power M-mode (upper panel) as a whitish sloping track. The whitish sloping track means that microemboli disrupt the ultrasonic signal (arrows).

(Figure 4). In the Spencer Logarithmic Scale Grades, grade I and Ⅱ are considered negative for patent foramen ovale, and grades Ⅲ through Ⅴ are considered positive. These findings indicate that the patient had a patent foramen ovale. Therefore, cerebral and splenic infarctions may develop due to emboli cause by a right-to-left shunt. At the follow-up examination, her neurologic symptoms were improved, but neurologic sequelae remained.

DISCUSSION

Endoscopic treatments, such as band ligation and injection sclerotherapy, became the cornerstone of the management of variceal bleeding. Endoscopic band ligation is the preferred form of endoscopic treatment for esophageal variceal bleeding, but its application in actively bleeding patients is still challenging because the bands at the endoscope tip limit the operator's field of vision. Injection sclerotherapy with various sclerosants is recommended in patients in whom endoscopic band ligation is not technically feasible. Several sclerosants are available, including 5% sodium morrhuate, 1%-3% sodium tetradecyl sulfate, 5% ethanolamine oleate, and 0.5%-1% polidocanol[2]. Adhesives such as Histoacryl have been used successfully for the treatment of variceal bleeding.

Injection sclerotherapy provides effective treatment for variceal bleeding, but it has been associated with a variety of complications. Minor complications including chest pain, dysphagia, fever, and esophageal ulcer are common, although not typically serious. Uncommon and serious complications include bacteremia, esophageal perforation, mediastinitis, and brain abscess. Rarely, systemic embolic complications have followed injection sclerotherapy, and these can be disastrous[4]. Systemic embolic complications following Histoacryl injection have been reported, and the common sites of embolic complications were the lung, spleen, cerebrum, and portal vein. Additionally, this complication has been principally described in the treatment of gastric variceal bleeding. Because most gastric varices are associated with a gastrorenal and splenorenal shunts[5], blood flow is abundant, and Histoacryl injection is likely to cause systemic embolization due to the migration of the agent through a shunt[6]. In contrast to gastric variceal injection, systemic embolic complications arising from esophageal variceal injection sclerotherapy are extremely rare; to date, only three cases of cerebral embolic complications following esophageal variceal injection sclerotherapy have been documented in the literature[7,8].

Because the bleeding esophageal varix was too large to apply band ligation, we performed injection sclerotherapy with Histoacryl. Cerebral and splenic infarctions followed the bleeding esophageal variceal injection sclerotherapy. Ours is an additional case of cerebral infarction caused by the esophageal variceal injection of Histoacryl, although it is the first report of a case of multiple embolizations including the cerebrum and spleen after the esophageal variceal injection of Histoacryl. The possible explanation for the development of systemic emboli may be the transient patent foramen ovale caused by the episodes of coughing, which induced a temporary right-to-left shunt.

Clearly, transesophageal echocardiography (TEE) is considered the gold standard for right-to-left shunt diagnosis, but it is poorly tolerated by patients and sometimes requires sedation. Additionally, TEE limits the patient's ability to perform a Valsalva maneuver[9]. Because our case had a large esophageal varix, we performed a TCD bubble test rather than TEE. The TCD bubble test has proven to be a trustworthy and less invasive method for diagnosing a right-to-left shunt[10]. In our case, the ultrasound waves were reflected by microbubbles on the TCD bubble

test, indicating the patent foramen ovale. Additionally, the TCD bubble test was grade Ⅲ during resting and grade Ⅳ during the Valsalva maneuver, according to the Spencer Logarithmic Scale[8]. Therefore, the cerebral and splenic infarctions in our case may have been caused by emboli via the patent foramen ovale.

Factors that increase embolization risk include the size of varices, the presence of a collateral vessel, excessive dilution, rapid polymerization, large volume (> 1 mL/injection) and rapid Histoacryl injection. Our case had the three possible embolic risk factors including the large size of the varices, the large volume (> 1 mL) of the mixture injected, and rapid injection[6]. Because most embolization risks associated with Histoacryl, as described above, are preventable, proper injection technique may help minimize the risk of serious complications and improve the long-term outcome.

Taken together, although injection sclerotherapy with Histoacryl is a relatively safe and efficacious procedure for the treatment of variceal bleeding, serious complications such as systemic embolization can occur. Ours is the first report of a case of multiple embolizations including the cerebrum and spleen after Histoacryl injection to treat esophageal variceal bleeding. Systemic embolization, despite its rarity, should be considered among the serious complications of Histoacryl injection for the treatment of esophageal variceal bleeding.

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CASE REPORT

Acute iatrogenic Budd-Chiari syndrome following hepatectomy for hepatolithiasis: A report of two cases

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Xue-Li Bai, Yi-Wen Chen, Qi Zhang, Long-Yun Ye, Yuan-Liang Xu, Liang Wang, Chun-Hui Cao, Shun-Liang Gao, Mohamed Adil Shah Khoodoruth, Bibi Zaina Ramjaun, Ting-Bo Liang, Department of Hepatobiliary-Pancreatic Surgery, Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310009, Zhejiang Province, China
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Abstract

Budd-Chiari syndrome (BCS) is defined as hepatic venous outflow obstruction at any level from the small hepatic veins to the junction of the inferior vena cava (IVC) and the right atrium, regardless of the cause of obstruction. We present two cases of acute iatrogenic BCS and our clinical management of these cases. The first case was a 43-year-old woman who developed acute BCS following the implantation of an IVC stent for the correction of stenosis in the IVC after hepatectomy for hepatolithiasis. The second case was a 61-year-old woman with complete obstruction of the outflow of hepatic veins during bilateral hepatectomy for hepatolithiasis. Acute iatrogenic BCS should be considered a rare complication following hepatectomy for hepatolithiasis. Awareness of potential hepatic outflow obstructions and timely management are critical to avoid poor outcomes when performing hepatectomy for hepatolithiasis.

Key words: Acute iatrogenic Budd-Chiari syndrome; Hepatolithiasis; Hepatectomy; Inferior vena cava

Core tip: The occurrence of acute iatrogenic Budd-Chiari syndrome (BCS) following hepatectomy for hepatolithiasis is rarely reported. However, it may occur following a particularly difficult hepatectomy for complicated hepatolithiasis. Here, we report two cases of acute BCS and present our clinical experience in managing these cases.

Bai XL, Chen YW, Zhang Q, Ye LY, Xu YL, Wang L, Cao CH, Gao SL, Khoodoruth MAS, Ramjaun BZ, Dong AQ, Liang TB. Acute iatrogenic Budd-Chiari syndrome following hepatectomy for hepatolithiasis: A report of two cases. *World J Gastroenterol* 2013; 19(34): 5763-5768 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i34/5763.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i34.5763

INTRODUCTION

Budd-Chiari syndrome (BCS) is characterized by hepatic venous outflow obstruction at the level of the hepatic venules, the large hepatic veins (HV), the inferior vena cava (IVC), or the right atrium[1]. Obstruction of the hepatic venous outflow tract, which leads to hepatic congestion as blood flows into, but not out of the liver, results in damage to the hepatic parenchymal cells and the cells lining the hepatic sinusoids. BCS can cause liver dysfunction and even lead to liver failure.

There are multiple known causes of BCS, including both heritable and acquired hypercoagulable states, compression or invasion of the IVC, as well as other less common miscellaneous causes[2-4]. BCS can also be idiopathic. Here, we report two cases of acute BCS with an uncommon cause and present our clinical experience in managing these cases.

CASE REPORT

Case 1

A 43-year-old woman developed acute BCS following the implantation of an IVC stent for correction of stenosis in the IVC after hepatectomy for hepatolithiasis. The patient was admitted with recurrent cholangitis. Magnetic resonance cholangiopancreatography showed lithiasis in the intra- and extra-hepatic bile duct, dilatation of the biliary tract and enlargement of the gallbladder. In addition, computed tomography (CT) (Figure 1) demonstrated right lobe, caudate lobe and segment Ⅱ (Counauid' s classification) atrophy of the liver. After biliary decompression by endoscopic retrograde cholangio-pancreatography and control of infection, the following procedures were performed: right hemi-hepatectomy, segment Ⅱ resection, caudate lobectomy, cholecystectomy, choledochotomy, and choledochojejunostomy. During right hepatectomy, the IVC was accidentally wedge resected due to the obscure boundary between the IVC and the right lobe of the liver, which was immediately sutured.

After surgery, the patient developed congestive hepatopathy, manifested as rapidly progressive abdominal distention, and severe pitting edema of the lower back and lower limbs. Magnetic resonance venography 3 d after operation (Figure 2) showed obvious stenosis near the entrance of the three HVs into the IVC, at the same location where the wedge was resected and repaired. Emergent balloon dilation was performed using a balloon catheter and a metallic stent (30 mm × 75 mm), guided by digital subtraction angiography (Figure 3). However, after this procedure, the patient's liver function deteriorated (Table 1). Contrast enhanced CT confirmed hepatic congestion and revealed that the proximal end of the stent was directly at the entrance of the HV into the IVC. Doppler ultrasonography showed reduced blood flow velocity in the HVs, the maximum velocity in the middle HV was 14 cm/s. There was turbulence in the left HV and backflow in the portal vein. Based on these findings, the patient was diagnosed with acute BCS, caused by the improper position of the stent which blocked the entrance of the HV into the IVC.

Following this diagnosis, the patient was immediately scheduled for surgery to remove the metallic stent. The stenosis of the IVC was 5 cm long and the diameter of the lumen was only 1 cm. The stenosis was repaired using a BalMedic (Beijing Balance Medical Co. Ltd, China) pericardial patch (2 cm × 5 cm) under cardiopulmonary bypass and deep hypothermic circulatory arrest (Figure 4). After surgery, the clinical manifestations of BCS

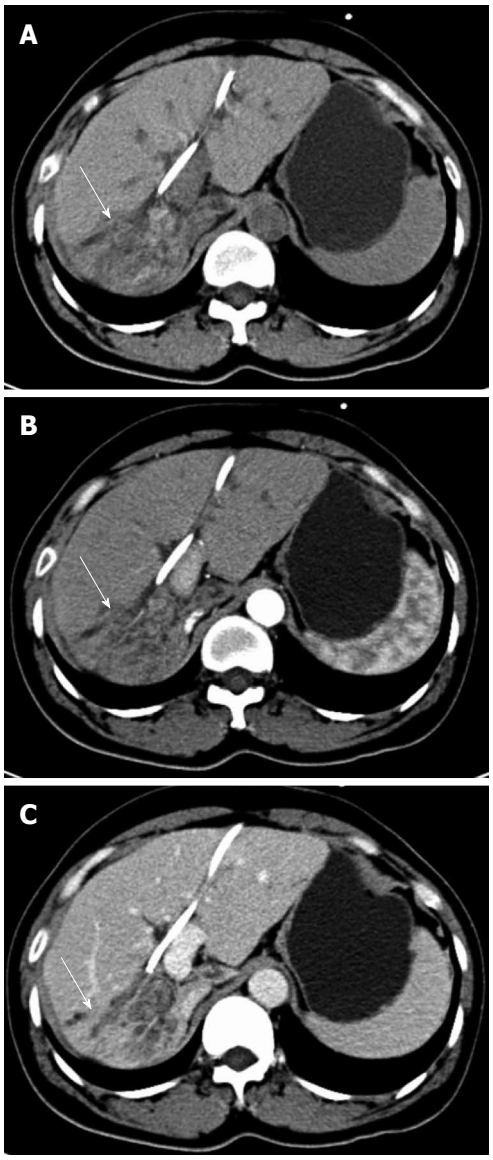

Figure 1 Preoperative computed tomography scan shows hepatolithiasis in the right liver with hepatic parenchymal atrophy (arrow). A: Non-contrast; B: Arterial phase; C: Venous phase.

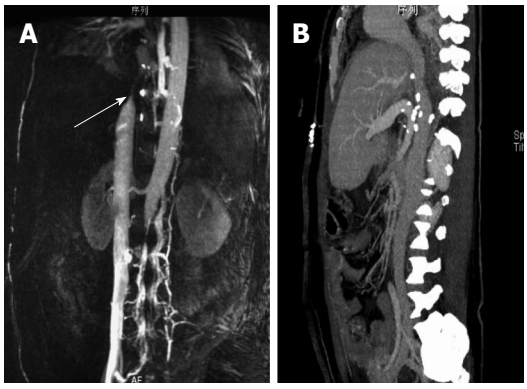

Figure 2 The inferior vena cava. A: Pretreatment of stenosis in inferior vena cava (IVC) on magnetic resonance venogram imaging (arrow); B: One year after patch repair, computed tomography angiography showed no obvious obstruction in the IVC and good hepatic vein outflow.

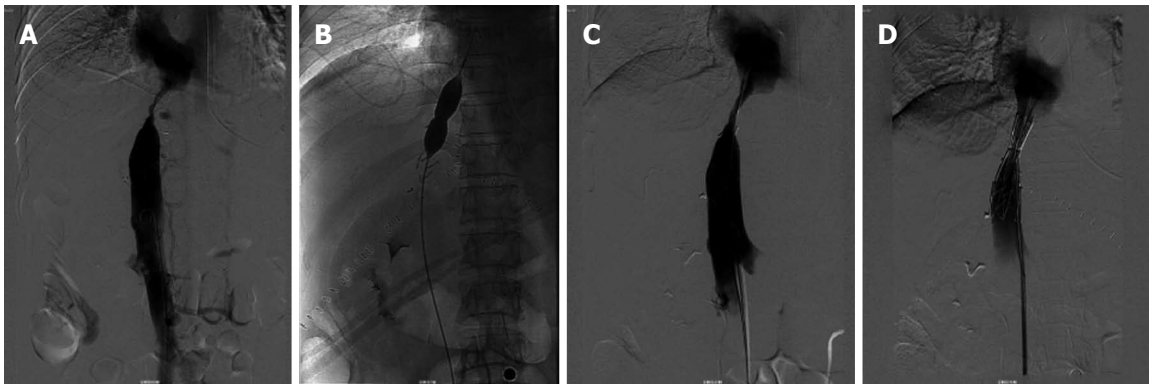

Figure 3 Treating stenosis of the inferior vena cava by balloon dilation and stent placement. A: Digital subtraction angiography shows the stenosis of inferior vena cava; B: Balloon dilation; C: After balloon angioplasty; D: Metallic stenting.

Table 1 Temporal liver function data for case 1

	Before hepatectomy	After hepatectomy	After metallic stent placement	5 d after IVC patch repair
TBIL (μmol/L)	26.7	45.2	83.3	26.3
DBIL (μmol/L)	13.6	34.3	45.6	17.3
IBIL (μmol/L)	13.1	15.8	37.7	9
ALT (U/L)	146	294	335	24
AST (U/L)	73	199	287	16
WBC (10^6/L)	4.0	10.3	5.9	6.0
Hb (g/L)	107	81	80	88
PLT (10^6/L)	117	51	57	73
PT (s)	11	18.4	20.5	16.0
INR	0.92	1.53	1.7	1.33

TBIL: Total bilirubin; DBIL: Direct bilirubin; IBIL: Indirect bilirubin; WBC: White blood cell; Hb: Hemoglobina; PLT: Platelet; PT: Prothrombin time; INR: International normalized ratio; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

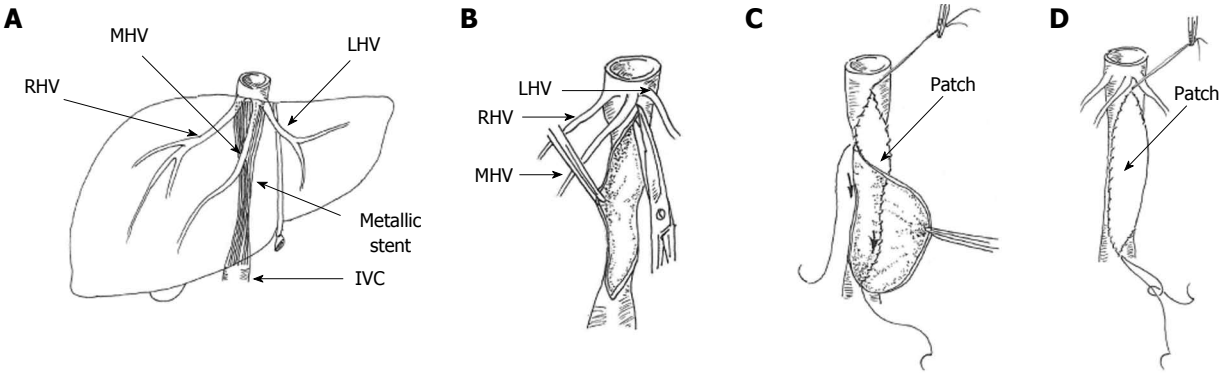

Figure 4 Diagram of the operative procedure for removing the metallic stent from the inferior vena cava and repairing the stenosis with a BalMedic pericardial patch. A: The proximal end of the stent was directly at the entrance of the hepatic veins into the inferior vena cava (IVC); B: Open the stenosis of IVC; C: BalMedic pericardial patch was anastomosed to the IVC to broaden its lumen; D: After patch repair of the IVC. LHV: Left hepatic vein; MHV: Middle hepatic vein; RHV: Right hepatic vein.

were alleviated, liver function improved, and the patient recovered without further complications (Table 1). Anticoagulation therapy with low molecular weight heparin (LMWH) for 3 d followed by Warfarin for 3 mo was administered. At one year follow-up, the patient had no recurrent cholangitis, no symptoms related to BCS, and normal liver function. CT angiography showed no obvious stenosis in the IVC (Figure 2).

Case 2

A 61-year-old woman experienced complete obstruction of the outflow of HVs during bilateral hepatectomy for hepatolithiasis. She underwent an open cholecystectomy and choledocholithotomy with T-tube drainage 15 years previously for cholelithiasis. In recent years, she suffered from recurrent cholangitis. Imaging findings (Figure 5) showed bilateral hepatolithiasis, dilation of the biliary

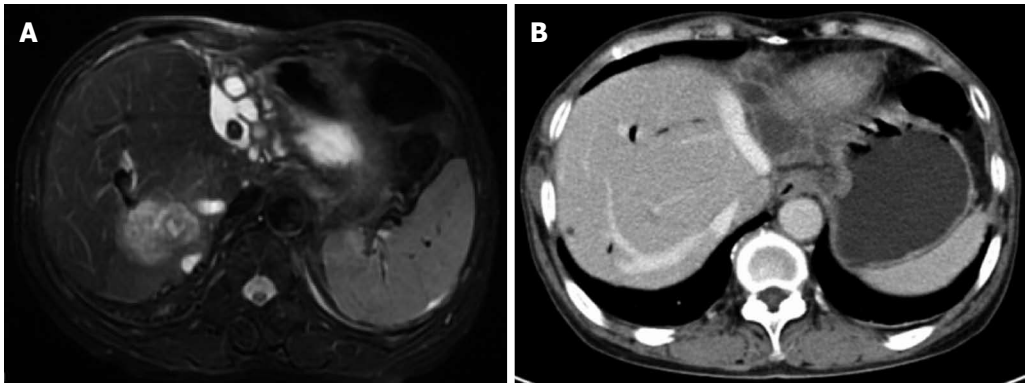

Figure 5 Preoperative imaging findings. A: Magnetic resonance imaging shows bilateral hepatolithiasis with infection at the posterior right lobe of the liver; B: Major hepatic vein on computed tomography.

tract, common bile duct stones, and a hypodense area on CT in the posterior lobe of the right liver, suggesting infection.

During surgery, the liver was found to be distorted with marked atrophy of the right posterior hepatic lobe and left half of the liver. In addition, the right anterior hepatic lobe (segment V and Ⅷ), and the right caudate lobe showed significant hyperplasia. No neoplasms were found in the wall of the bile duct during choledochoscopy. The liver was then freed to begin resection of the left part of the liver, the right posterior lobe and the right caudate lobe. The left half was resected first. Then, the right HV was revealed and divided using a 45-mm endoscopic linear stapler (Echelon 45 ENDOPATH Stapler, Ethicon ENDO-Surgery, Cincinnati, Ohio, United States). Immediately after ligation, there was sudden and significant congestion of the liver, and hepatic outflow blockage was suspected. The liver was examined and stenosis was found in the middle HV, which was thought to be the left HV. Thus, we set out to repair the middle HV and the right HV.

The stenosed middle HV was opened and cryopreserved (-80 ℃). A common iliac artery allogenic graft about 3 cm was prepared. The lumen of the artery was opened and anastomosed to the MHV forming a patch to broaden the stenosed middle HV. Liver swelling was observed to subside after middle HV reconstruction. Both ends of the right HV were then clamped using vascular clamps to control bleeding, and a 1-2 cm allogeneic iliac vein graft was used to repair the right HV (Figure 6). The outflow of blood from the liver was then assessed; no thrombosis or air embolism was found and the liver swelling subsided. It should be noted that the hepatic blood inflow of the porta hepatis was blocked several times during this procedure. The longest blockage time was 40 min with a total length of 60 min. During the procedure, the liver was cold conditioned using ice to attenuate warm ischemic injury. A T-tube was placed in the common bile duct for drainage and to improve accessibility for the future removal of residual stones in the biliary tract. Due to the episode of HV obstruction, a right posterior lobectomy was not performed.

Postoperatively, peak liver enzymes aspartate aminotransferase 380 U/L and alanine aminotransferase 407 U/L were detected, with normal total bilirubin at postoperative day 2. Liver enzymes decreased to the normal range within one week. Postoperative recovery was uneventful. Anticoagulation therapy with LMWH for 3 d followed by Warfarin for 3 mo was administered. Postoperative CT scan (Figure 7) showed no obstruction or thrombosis in the right or middle HV.

DISCUSSION

BCS is a rare clinical condition characterized by hepatic venous outflow obstruction due to various causes[1]. The most common causes of BCS are hypercoagulable states, other uncommon causes have been identified such as tumor invasion, otherwise its origins are idiopathic[3]. There are also sporadic reports of BCS as a surgical complication after liver transplantation[5], and right hepatectomy resulting from torsion of the remnant liver[6]. In this study, we report two cases of acute BCS following hepatectomy for hepatolithiasis.

Hepatolithiasis is a common disease in Asian countries[7]. A multimodal approach has been advocated in the management of this condition, including endoscopic, percutaneous, or open surgery. Hepatectomy is considered the most effective treatment and is indicated in some cases, especially those with recurrent bacterial cholangitis and irreversible atrophy of parts of the liver[8-10]. Despite the efficacy of this approach, higher morbidity and mortality is associated with hepatectomy for hepatolithiasis[9]. As observed in the two reported cases, performing a partial hepatectomy for hepatolithiasis is a particularly difficult and challenging procedure because of the dense perihepatic adhesions (due to recurrent cholangitis or previous surgery), and abnormal intrahepatic anatomy (due to the atrophy-hypertrophy complex)[11]. These challenges require detailed preoperative evaluation to avoid venous injury, including high resolution imaging to identify the intrahepatic anatomy, as well as the relationship between the liver and the IVC. In addition, due to coexisting atrophy and regeneration of the liver, the func-

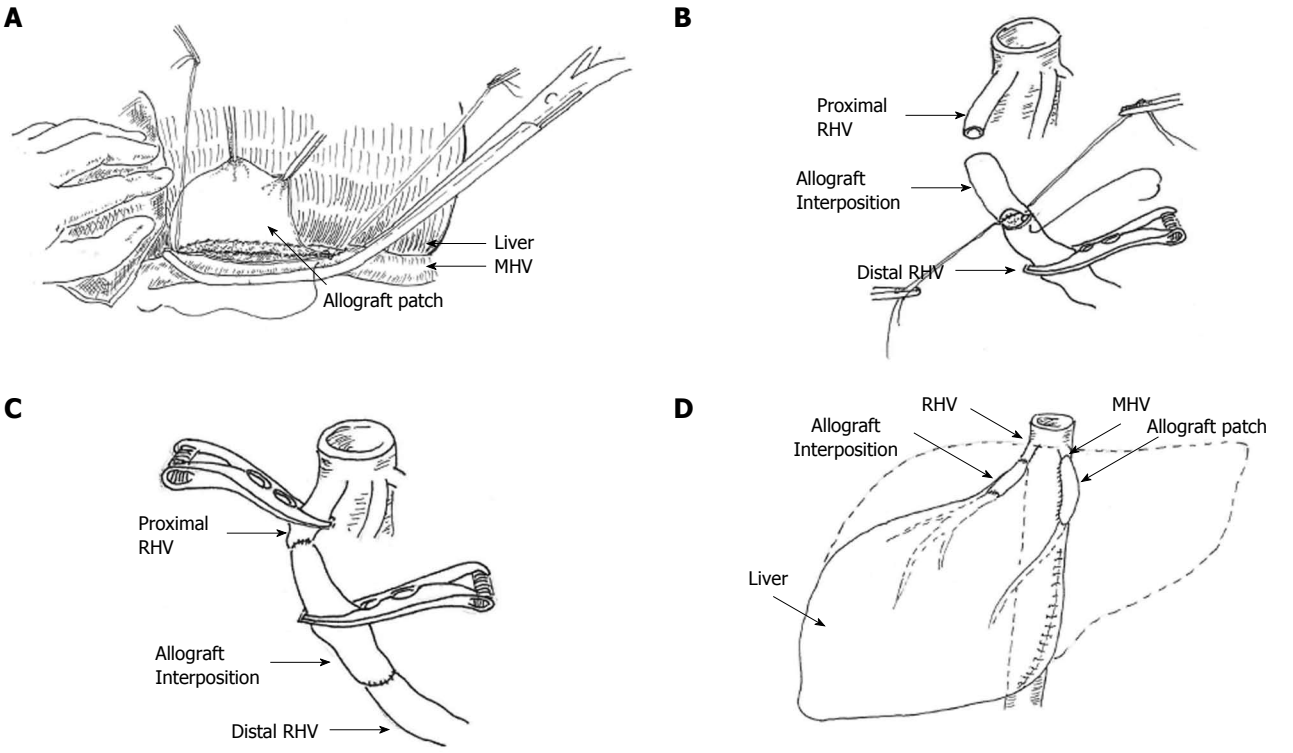

Figure 6 Diagram of reconstruction of middle and right hepatic vein. A: Middle hepatic vein (MHV) reconstruction using a common iliac artery allogenic graft; B: Right hepatic vein (RHV) reconstruction using an allogeneic iliac vein graft; C: Completion of RHV reconstruction using allograft interposition; D: Accomplishment of MHV and RHV outflow.

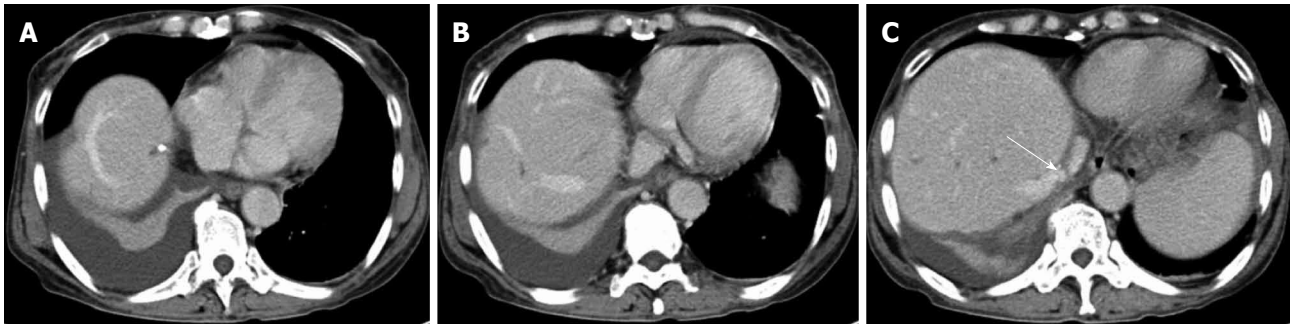

Figure 7 Postoperative computed tomography scan. A, B: Demonstrated good right hepatic vein tract; C: The inter-positioned graft using an allogeneic iliac vein (arrow).

tion of the HV is frequently abnormal, thus it is crucial to ligate the major HV for testability before continuing with the procedure. Based on our experience with hepatic outflow obstruction, we believe that awareness of possible complications and timely management are critical for attenuating liver congestion and salvaging the liver.

The treatment of BCS is based on its cause, consisting of anticoagulation therapy, thrombolytic therapy, radiological procedures (*i.e.*, angioplasty, stenting or transjugular intrahepatic portosystemic shunts), surgical decompression, surgical shunts, and surgical correction of the lesion. Rarely, liver transplantation may be necessary if the liver is irreparably damaged[12,13]. Of note, balloon angioplasty and stent placement has been increasingly favored and the effectiveness of these procedures has been documented[1,14]. However, in the first case presented above, the stenosis was very close to the secondary porta of the liver, and the placement of the stent was incorrect as it blocked the entrance of the HV into the IVC. Thus, based on our experience, surgical broadening of the stricture of the IVC is strongly advocated. With regard to our second case, it is evident that no other method is a substitute for surgical reconstruction of the HV to resolve hepatic congestion.

In conclusion, episodes of acute iatrogenic BCS following hepatectomy for hepatolithiasis are a rare occurrence. Awareness of potential hepatic outflow obstructions and timely management are critical to avoid poor outcomes when performing hepatectomy for hepatolithiasis. From our experience, prompt surgical reconstruction of the HV should be favored to salvage the congested liver.

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11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6

24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23243641.

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Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
Biology: *H. pylori*, *E coli*, *etc.*

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