ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

World Journal of *Gastroenterology*

World J Gastroenterol 2012 December 7; 18(45): 6521-6692

Baishideng Publishing Group
www.wjgnet.com

WJG World Journal of Gastroenterology

A peer-reviewed, online, open-access journal of gastroenterology and hepatology

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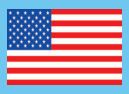

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World Journal of Gastroenterology

Contents

Weekly Volume 18 Number 45 December 7, 2012

World Journal of Gastroenterology
Volume 18 Number 45 December 7, 2012

Contents

NAME OF JOURNAL
World Journal of Gastroenterology

ISSN AND EISSN
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

RESPONSIBLE INSTITUTION
Department of Science and Technology of Shanxi Province

SPONSOR
Taiyuan Research and Treatment Center for Digestive Diseases, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China

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PUBLISHER
Baishideng Publishing Group Co., Limited
Room 1701, 17/F, Henan Building,
No.90 Jaffe Road, Wanchai, Hong Kong, China
Fax: +852-31158812
Telephone: +852-58042046
E-mail: bpgoffice@wjgnet.com
http://www.wjgnet.com

PRINT SUBSCRIPTION
RMB 300 Yuan for each issue, RMB 14400 Yuan for one year.

PUBLICATION DATE
December 7, 2012

INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm

ONLINE SUBMISSION
http://www.wjgnet.com/esps/

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v18.i45.6521

World J Gastroenterol 2012 December 7; 18(45): 6521-6526
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

EDITORIAL

Esophageal malignancy: A growing concern

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Received: March 24, 2012 Revised: May 19, 2012
Accepted: June 8, 2012
Published online: December 7, 2012

Abstract

Esophageal cancer is mainly found in Asia and east Africa and is one of the deadliest cancers in the world. However, it has not garnered much attention in the Western world due to its low incidence rate. An increasing amount of data indicate that esophageal cancer, particularly esophageal adenocarcinoma, has been rising by 6-fold annually and is now becoming the fastest growing cancer in the United States. This rise has been associated with the increase of the obese population, as abdominal fat puts extra pressure on the stomach and causes gastroesophageal reflux disease (GERD). Long standing GERD can induce esophagitis and metaplasia and, ultimately, leads to adenocarcinoma. Acid suppression has been the main strategy to treat GERD; however, it has not been proven to control esophageal malignancy effectively. In fact, its side effects have triggered multiple warnings from regulatory agencies. The high mortality and fast growth of esophageal cancer demand more vigorous efforts to look into its deeper mechanisms and come up with better therapeutic options.

Key words: Esophageal cancer; Gastroesophageal reflux disease; Obesity

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Chai J, Jamal MM. Esophageal malignancy: A growing concern. *World J Gastroenterol* 2012; 18(45): 6521-6526 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i45/6521.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i45.6521

INTRODUCTION

While the incidence of most cancers is declining, esophageal cancer has been continuing its march as the fastest growing malignancy in the Western world[1,2]. This rise is largely derived from gastroesophageal reflux disease (GERD), which is associated with the proliferation of obesity. The continuous growth of the obese population foreshadows a future increase of GERD and its associated esophageal cancer. This demands immediate and more rigorous research on the molecular mechanisms of this common disease and its pathways which lead to esophageal malignancy. Current GERD treatment mainly relies on acid suppression drugs which have not been proved to change the risk of cancer development. Although the debate is still going on whether gastric acid or bile acid is ultimately responsible for GERD malignancy, based on the data from human studies, animal modeling, and *in vitro* simulation, perhaps it is time to explore other options.

STATISTICS OF ESOPHAGEAL CANCER: RISING NUMBERS

Cancer is the second leading cause of death in the world[3]

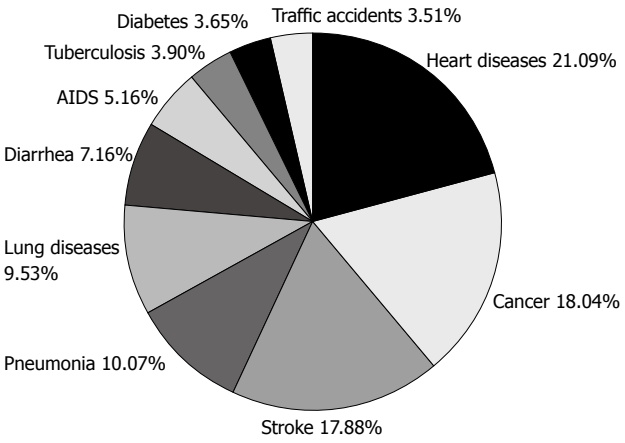

Figure 1 Top 10 leading causes of death worldwide. Cancer is the second highest one. Data extracted from World Health Organization documents. AIDS: Acquired immune deficiency syndrome.

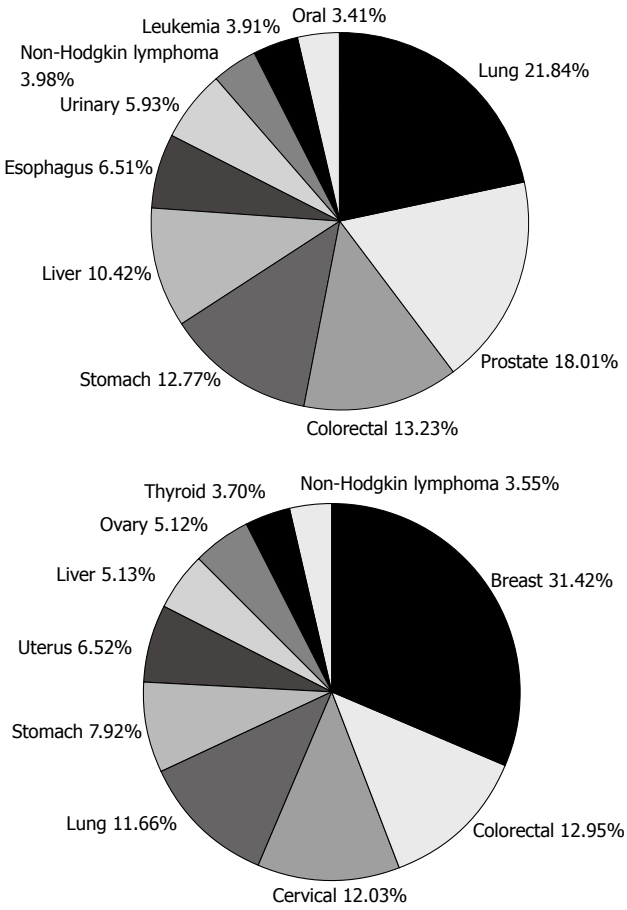

Figure 2 Top 10 of the most common cancers in men (up) and women (below) worldwide. Esophageal cancer is No. 7 in men. Data extracted from World Health Organization documents.

after heart disease (21.09%), contributing 18.04% to the total number of deaths worldwide (Figure 1). Among all types of cancers, skin cancer is the most common one, which includes 2-3 million non-melanoma and 132 000 melanoma cases diagnosed each year, making up one third of the total cancer cases. According to Skin Cancer Foundation Statistics, one in every five Americans will develop skin cancer in their lifetime. This prevalence is largely due to depletion of ozone in the atmosphere, which weakens our planet's protective shield from the brunt of the sun's harmful rays. It is estimated that every 10% decrease in ozone levels will generate an additional 300 000 non-melanoma and 4500 melanoma cancer cases. However, the majority of skin cancer can be easily treated, while digestive cancers, such as esophageal cancer, are highly life-threatening.

Esophageal cancer is found more commonly in males than in females, with a ratio of approximately 7:1. Currently, it is the 7th leading cancer in men globally, contributing 6.51% to the total number of male cancer cases (Figure 2). There are two main subtypes of esophageal cancer: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). ESCC occurs most often in the middle portion of the esophagus and accounts for 90%-95% of all cases of esophageal cancer worldwide, while EAC is primarily found in the lower esophagus. The type and incidence of esophageal cancer varies dramatically depending on the geographical location (Figure 3). The top 10 countries with the highest age-standardized death rate due to esophageal cancer are Nauru (30.3), Sao Tome (26.4), Mongolia (18.6), South Africa (18.2), Malawi (18.2), China (15.5), Lesotho (15.5), Kenya (13.9), Mozambique (13.5) and Uganda (13.4) (the death rate being deaths per 100 000 people). The highest rates are found in Asia, stretching from northern Iran through the central Asian republics to north-central China, often referred to as the "esophageal cancer belt". For instance, in China, the majority of esophageal cancer diagnoses are ESCC and it is ranked as the 8th leading cause of death nationwide (Table 1), mostly in northern China where the incidence rate can be as high as 800 cases per 100 000 people. On the other hand, in the United States, more than 50% of esophageal cancer cases are EAC, and the rate is less than 5 in 100 000, making it the 29th leading cause of death. For this reason, esophageal cancer is not even on the current list of common cancers in the United States, according to the National Cancer Institute. In order to be on the list, esophageal cancer has to have at least 40 000 cases a year, while the current estimate for 2012 is only 17 460. Although the reason for this geographic variation still needs investigation, several factors have been suggested that might all contribute to the issue to a certain degree, such as *Helicobacter* infection, dietary pattern, and life habits[4]. As Eastern and Western countries become increasingly open to each other and people adapt more to each other, we expect this discrepancy will become less and less. As evident in China, EAC incidence was doubled from the 1970s to the 1980s, according to an examination of the medical records of esophageal cancer patients diagnosed from 1970 to 2001 in a local hospital[5].

These numbers only tell one side of the story. Based on the annual reports on the status of cancer[1,2], although esophageal cancer is low in Americans, it has been rising by 6-fold annually and its increase rate now exceeds that

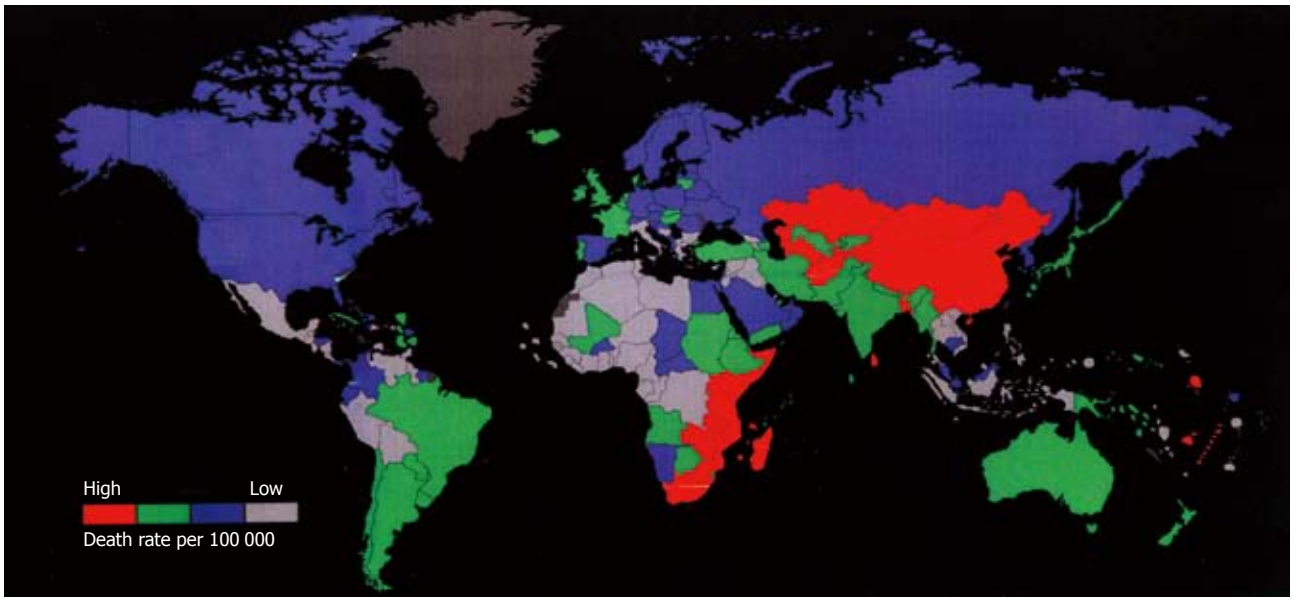

Figure 3 Geographic distribution of esophageal cancer. China is a hot spot. Data from World Health Organization documents.

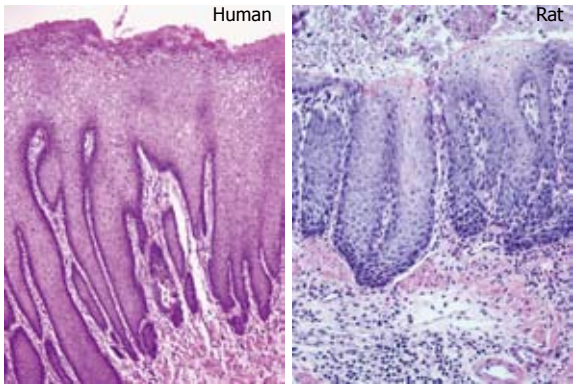

Figure 4 Gastroesophageal reflux disease-induced esophagitis in human and rat (hematoxylin and eosin staining). Gastroesophageal reflux disease rats were created by surgically anastomosing the duodenum to the gastroesophageal junction. These rats can develop esophageal adenocarcinoma within a year, in a pathological sequence similar to human esophageal malignancy.

for any other type of cancer. There are several possible reasons for this rise, such as excessive alcohol consumption, smoking, stress, and a diet low in vegetables, but the leading factor is GERD, a term that frequently appears in the media as well as in general conversations. A recent study showed that GERD increases the risk of esophageal cancer by 8.6 fold[6].

GERD: NOT A SMALL PROBLEM

GERD is the most common gastrointestinal diagnosis given during office visits and its direct medical costs, which primarily include drug costs, exceed $10 billion a year in the United States[7], whereas indirect costs resulting from reduced work productivity are estimated to be as much as $75 billion a year[8]. GERD occurs when the esophageal sphincter at the bottom of the esophagus weakens and allows stomach acid (often mixed with duodenal contents) to back up into the esophagus. The refluxate erodes the epithelial lining of the lower esophagus and gives a burning sensation in the middle of the chest, which is commonly described as "heartburn". Patients with long standing GERD can develop esophagitis, an inflammation characterized histologically by a markedly thickened epithelium, elongation of the lamina propria papillae into the epithelium, and basal cell hyperplasia (Figure 4). Over time, this inflammation/injury cycle can induce esophageal mucosa transformation from squamous to a more protective intestinal columnar phenotype, known as Barrett's esophagus (BE). From a physiological point of view, the secretory columnar epithelium is better prepared to withstand the erosive action of the acidic refluxate than squamous epithelium; however, this metaplastic change confers an increased risk of transformation to EAC. Studies have shown that people with BE can have as high as a 400-fold increased risk of EAC[9,10]. Today, over 60% of Americans experience occasional episodes of acid reflux, and about 25% deal with the problem on a weekly basis. The prevalence of the condition in Americans is increasing by approximately 5% annually[11]. Hospitalizations for all GERD-caused esophageal disorders doubled from 1998 to 2005, according to the United States Agency for Healthcare Research and Quality.

OBESITY: THE DEVIL

The rise in GERD is associated with the rapidly growing obese population[12], which is usually measured by body mass index (BMI). BMI is calculated based on the weight and height of a person [BMI = weight/(height)^2, kg/m^2]. The World Health Organization regards a BMI of less than 18.5 as underweight and may indicate malnutrition, an eating disorder, or other health problems, while a BMI greater than 25 is considered overweight and above 30 is considered obese. A recent study showed that global obe-

Table 1 Top 10 leading causes of death in China *vs* the United States

Rank	China			United States		
	Diseases	Deaths	Death rate (%)	Diseases	Deaths	Death rate (%)
1	Stroke	2 125 802	23.92	Coronary heart disease	445 864	21.42
2	Lung disease	1 287 089	14.48	Alzheimer/dementia	172 765	8.30
3	Coronary heart disease	1 040 692	11.71	Lung cancers	165 402	7.95
4	Lung cancers	460 856	5.19	Stroke	146 664	7.05
5	Liver cancer	380 491	4.28	Lung disease	130 808	6.29
6	Stomach cancer	354 829	3.99	Diabetes mellitus	75 280	3.62
7	Road traffic accidents	292 481	3.29	Colorectal cancers	62 592	3.01
8	Esophageal cancer	212 537	2.39	Hypertension	62 156	2.99
9	Other injuries	209 836	2.36	Pneumonia	57 722	2.77
10	Hypertension	205 689	2.31	Kidney disease	50 889	2.45

Esophageal cancer is the No. 8 killer in China, while in the United States, it is No. 29. Here the death rate is the percentage of the total deaths nationwide. Data from World Health Organization, World Bank and National Institute of Health.

sity rates have doubled since 1980[13]. The health care costs resulting from excess weight are estimated at greater than $100 billion annually in the United States. According to the report released last year from the Centers for Disease Control and Prevention, more than 34% of adult Americans are obese, which is higher than Canadians (24%). It is predicted that by the year 2020, 77.6% of men will be overweight and 40.2% obese; the corresponding figures for women will be 71.1% and 43.3%, respectively[14]. This problem has also affected children, whose obesity rate has tripled in the last 30 years[15]. At the time of writing, 15.5% of children in the United States are obese. A recent study with a study population of 690 321 patients (age: 2-19 years) revealed that obese children have a 30%-40% higher risk of GERD, compared with children with a normal weight according to their BMI (BMI = 20 ± 3.8 kg/m^2)[16]. In adults, the situation is worse. In 2007, a study showed that the total number of GERD episodes was 48% higher in obese patients than those with a normal BMI[17]. The link between increasing BMI and the presence of GERD was further strengthened by a meta-analysis of 20 independent studies, which established a dose-dependent association between these two conditions[18]. A similar connection has also been drawn between increasing BMI and esophageal cancer[19-23].

The precise pathophysiological pathway from obesity to GERD has not been fully elucidated. It has been shown that excess fat in the abdominal area can push on the stomach's contents to back up, relax the lower esophagus muscle[24,25], disable the esophageal motor[26], impair stomach accommodations[27], and ultimately result in a higher frequency of esophageal acid exposure[12,28,29]. Therefore, a potential causal pathway from body size to esophageal cancer may be from normal to GERD to esophagitis to BE, and ultimately to EAC. In such a direct pathway, obesity could act by increasing the prevalence of GERD, by increasing the prevalence of BE among the GERD population, or by enhancing the risk of malignant transformation from BE to EAC. Although obesity is a major contributor, other factors (e.g., smoking, drinking, diet, or genetics) may also influence the steps on this pathway. For example, GERD smokers were found to have 12.3-fold higher risk of developing EAC than GERD non-smokers[6]. While the issue is quite complex, since the main pathway starts with GERD, interventions aimed at GERD should be expected to proportionally lower the risk of the subsequent steps in the pathway: BE and EAC.

TREATMENT: NO WINNERS

Current treatment for GERD patients includes acid suppressive medications and lower esophageal repair surgery. Aside from traditional antacids (Alka-Seltzer and Tums) which have side effects such as diarrhea and constipation, there are now two categories of medications to treat GERD. H-2 blockers (e.g., Zantac 75, Pepcid AC, Tagamet HB and Axid AR) reduce the amount of histamine-2, which produces acid in the stomach, and are recommended for people with less frequent/severe heartburn. A second medication is the proton pump inhibitors or proton pump inhibitors (PPIs) (e.g., Prilosec, Prevacid, Protonix and Nexium), which directly shuts down the H^+/K^+ ATPase pump of the parietal cells in the stomach that produce acid. These drugs are stronger than H2 blockers and are recommended for people with more persistent/acute symptoms. Control of acid reflux with PPIs has been found extremely effective for healing reflux esophagitis, but not for prevention of BE development or its progression to EAC. As matter of fact, more and more evidence is emerging about the long-term side-effects associated with these drugs, such as decreased absorption of vitamins/minerals[30], susceptibility to bacterial infections[31], bone fracture[32], and even elevated risk of developing EAC[6,33]. The Food and Drug Administration of the United States has issued warnings repeatedly over the years on high-dose or long-term use of PPIs.

For people who have responded to medication but continue to experience GERD symptoms, surgery to reconstruct the lower esophageal sphincter is usually an option. However, only about 5% of GERD patients undergo surgery and a follow-up study showed that almost two-thirds of the surgical patients were back on medica-

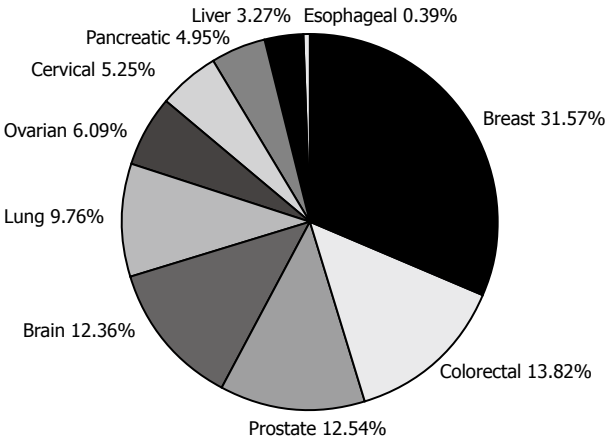

Figure 5 National Institute of Health expenditure on cancer-related studies in 2011. Esophageal cancer barely shows on the pie chart. Data from National Institute of Health documents.

tion[34]. A more recent study reported that although surgical therapy achieved better remission of GERD symptoms than Prilosec, 36% of surgical-treated GERD patients ultimately received PPI medication, while 14% of PPI-treated GERD patients underwent subsequent surgery[35]. One final way to treat GERD is through endoscopic procedures, including stitching or using radio-frequency waves to reconstruct the lower esophageal sphincter, but in 2002 this was not recommended by the American Gastroenterological Association for GERD treatment.

CAUSE OF EAC: ACID OR BILE? WHAT TO BLAME?

The inadequacy of treatment options raises the question on the real scientific basis of acid suppression in GERD treatment. A direct chemical analysis of esophageal fluid showed that GERD patients contain about 10 times more bile acid in their lower esophagus than normal people[36]. This might give us a clue as to why regular use of acid suppressants has not lowered the risk of GERD malignancy. In support of this notion, animal studies showed that gastric reflux alone does not cause EAC at all; it is the duodenal contents per se that ultimately lead to esophageal malignancy[37-39]. Furthermore, some animal studies even suggested that gastric acid may play a protective role in GERD against malignancy. For example, one study showed that 87% of rats with surgically-created duodenal reflux alone developed EAC, while the percentage of rats with gastric-duodenal reflux was only 30%[40]. In agreement with the animal studies, a recent systematic review[41,42] examined publications indexed in MEDLINE from 1950 to 2010, and found 82 original human studies on the association of bile acids with GERD, among which, all *in vivo* studies detected bile acids in the esophageal aspirates of GERD patients, and what's more, their concentrations were significantly higher than in normal people. It is clear that the refluxate of GERD patients frequently contains bile acids, sometimes even in millimolar concentrations. In addition to human studies and animal modeling, *in vitro* experiments have shown that bile acids, at equivalent concentrations to the ones found in the esophagus of GERD patients, can stimulate esophageal epithelial cells to produce inflammatory cytokines and chemokines, to generate reactive oxygen species, and to express intestinal genes. All these factors have the ability to facilitate esophageal epithelial metaplasia and even malignancy.

CONCLUSION

Although skin cancer is the most common cancer in the world, 95% of cases are either basal cell carcinoma or squamous cell carcinoma, which have less than 0.5% mortality. Even for the most deadly type of skin cancer - melanoma, which is very rare - the death rate is only about 15%. So is prostate cancer, the second most common cancer in the United States. On the other hand, although esophageal cancer patients have a mortality rate of approximately 85%, since it is less common than either skin cancer or prostate cancer, it receives little attention from government agencies or the research community. This can be seen by looking at the annual budget of the National Institute of Health (NIH) of the United States. In 2011, the NIH spent $284 million on 772 prostate cancer research projects, while only funding 30 esophageal cancer studies with $13 million (Figure 5). Through this article, we hope to attract attention to this disease since, due to its high mortality and fast growth, esophageal cancer could be catastrophic in the near future if we do not prepare ourselves with the proper knowledge.

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S- Editor Wu X **L- Editor** Rutherford A **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v18.i45.6527

World J Gastroenterol 2012 December 7; 18(45): 6527-6531
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

FIELD OF VISION

Does antecolic reconstruction decrease delayed gastric emptying after pancreatoduodenectomy?

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Received: September 17, 2012 Revised: November 4, 2012
Accepted: November 11, 2012
Published online: December 7, 2012

Abstract

Delayed gastric emptying (DGE) is a frequent complication after pylorus-preserving pancreatoduodenectomy (PpPD). Kawai and colleagues proposed pylorus-resecting pancreatoduodenectomy (PrPD) with antecolic gastrojejunal anastomosis to obviate DGE occurring after PpPD. Here we debate the reported differences in the prevalence of DGE in antecolic and retrocolic gastro/duodeno-jejunostomies after PrPD and PpPD, respectively. We concluded that the route of the gastro/duodeno-jejunal anastomosis with respect to the transverse colon; i.e., antecolic route or retrocolic route, is not responsible for the differences in prevalence of DGE after pancreatoduodenectomy (PD) and that the impact of the reconstructive method on DGE is related mostly to the angulation or torsion of the gastro/duodeno-jejunostomy. We report a prevalence of 8.9% grade A DGE and 1.1% grade C DGE in a series of 89 subtotal stomach-preserving PDs with Roux-en Y retrocolic reconstruction with anastomosis of the isolated Roux limb to the stomach and single Roux limb to both the pancreatic stump and hepatic duct. Retrocolic anastomosis of the isolated first jejunal loop to the gastric remnant allows outflow of the gastric contents by gravity through a "straight route".

Key words: Antecolic reconstruction; Retrocolic reconstruction; Pancreatoduodenectomy; Pylorus-preserving pancreatoduodenectomy; Delayed gastric emptying

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Peparini N, Chirletti P. Does antecolic reconstruction decrease delayed gastric emptying after pancreatoduodenectomy? *World J Gastroenterol* 2012; 18(45): 6527-6531 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i45/6527.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i45.6527

INVITED COMMENTARY ON HOT ARTICLES

Delayed gastric emptying (DGE) is a major cause of early morbidity following pancreatoduodenectomy (PD). Although it has been recently reported that pylorus-preserving pancreatoduodenectomy (PpPD) and classical Wipple's PD are equal operations regarding the postoperative development of DGE[1], the occurrence of this complication is usually considered to be associated with PpPD. DGE after PpPD was first described by Warshaw *et al*[2] in 1985. DGE implies a state of postoperative gastroparesis and gastric stasis for which prolonged gastric drainage is necessary with delay to return to solid food intake. However, the pathogenesis of DGE is still unclear. Postoperative decrease in plasma motilin stimulation after duodenal resection[3], devascularization and denervation of the pylorus with subsequent pylorospasm in PpPD[4,5] and other operative factors such as the route of gastro- or

duodeno-enteric reconstruction (antecolic *vs* retrocolic)[6] and the type of reconstructive technique (Billroth I *vs* Billroth II reconstruction)[7] may contribute to the occurrence of DGE. Moreover, intra-abdominal postoperative complications such as pancreatic fistula, peripancreatic collections, intraabdominal abscess or postoperative pancreatitis may increase the prevalence of DGE[8-13]. The reported prevalence of DGE after pancreatic surgery is remarkably variable due to different adopted definitions of DGE[10,14,15]. In fact, a consensus definition of DGE based on the impact on the clinical course and on postoperative management was proposed by the International Study Group of Pancreatic Surgery only in 2007[16]. Kawai *et al*[17] reported a prospective randomized controlled trial (RCT) on the prevalence of DGE in pylorus-resecting pancreatoduodenectomy (PrPD) *vs* PpPD. The authors proposed PrPD, in which the stomach is nearly entirely preserved and divided just adjacent to the pyloric ring, to obviate DGE occurring after PpPD and avoid the impairment of nutritional status occurring after classical Wipple's PD. They highlighted that the results of their RCT significantly favored PrPD over PpPD, considering the prevalence of DGE (4.5% *vs* 17.2%): in these procedures an antecolic gastro- or duodeno-jejunal reconstruction was adopted[18]. A recent RCT comparing the occurrence of DGE after subtotal stomach-preserving pancreatoduodenectomy in pancreaticogastrostomy with retrocolic gastro-jejunostomy reconstruction and in pancreaticogastrostomy with antecolic gastro-jejunostomy reconstruction concluded that antecolic reconstruction, and not retrocolic reconstruction, decreases DGE prevalence. However, in this study, Billroth I (retrocolic) reconstructions were compared with Billroth II (antecolic) reconstructions[19]. After subtotal stomach-preserving pancreatoduodenectomy with pancreaticogastrostomy, Oida *et al*[20,21] considered retrocolic gastrojejunal reconstruction preferable to antecolic reconstruction for preventing DGE because pancreaticogastric anastomosis is located behind the stomach and the retrocolic route in gastroenteric reconstruction enables the gastric contents to easily reach the jejunum. In the study by Eshuis *et al*[22], DGE was more frequent in retrocolic reconstructions, but in multivariable analysis no association between the route of reconstruction and DGE was found.

After PD, Billroth I reconstruction is considered to have a higher incidence of DGE than Billroth II reconstruction[7], but Billroth I is considered to be a more physiologic procedure than Billroth II because Billroth I preserves the proximal jejunum in the alimentary circuit and maintains the hormonal stimuli on the remnant pancreas[23]. In evaluation of the prevalence of DGE in antecolic and retrocolic reconstruction in gastro- and duodeno-jejunostomy after classical Wipple's PD and PpPD, respectively, the two compared procedures should differ only in the manner in which the jejunum is brought up in respect to the transverse colon. Kawai participated in a previously reported prospective RCT in which the adopted reconstructive procedures after PpPD were different only regarding the route; i.e., antecolic or retrocolic, for Billroth II type duodeno-jejunal anastomosis. The prevalence of DGE was significantly lower in the antecolic duodeno-jejunostomy group than in the retrocolic duodeno-jejunostomy group[6]. However, another recent RCT showed no difference in the prevalence of DGE between antecolic and retrocolic gastro/duodeno-jejunostomy following classical Wipple's PD/PpPD after standardization of both the antecolic and retrocolic types of Billroth II gastro/duodeno-jejunostomy with respect to the distance from the hepatico-jejunostomy and angulation of the jejunal loop. In this study, the occurrence of DGE was not affected by the type of performed PD; i.e., classical Wipple's PD *vs* PpPD, or the type of adopted reconstruction of the gastro/duodeno-jejunostomy; i.e., antecolic *vs* retrocolic[24]. Ueno *et al*[25] indicated that the transient torsion or angulation in the reconstruction of the alimentary tract is the main cause of DGE after PpPD. Several methods were proposed to promote the alimentary transit from the stomach through the jejunal loop, such as alignment of the stomach contour to avoid angulation of the jejunal loop distally to the duodeno-jejunal anastomosis in a Billroth I type of reconstructive procedure[25], and straight antecolic duodeno-jejunostomy twisting the jejunum 30° counterclockwise to preserve the patency of the efferent jejunum and placing the stomach in the left subcolic fossa to straighten it in a Billroth II type of reconstruction[26]. In the RCT by Chijiiwa *et al*[27] no significant difference in the prevalence of DGE was found between retrocolic vertically performed duodenojejunostomy and antecolic duodenojejunostomy (Table 1).

Regarding the resection method, Kawai *et al*[17,18] highlighted that PrPD preserves the capacity of the stomach and obviates to pylorospasm, denervation and devascularization of the pylorus ring, which can occur in PpPD, and demonstrated that PrPD decreases the incidence of DGE in respect to PpPD. Recently, these surgical procedures of subtotal stomach-preserving (or pylorus-resecting) pancreatoduodenectomies have been adopted in surgical treatments of malignant tumors of the periampullary region and of the head of the pancreas. Our group has been adopting subtotal stomach-preserving pancreatoduodenectomy since 1995 for several considerations. After pancreaticoduodenectomy, gastric preservation favors adequate weight gain due to higher caloric intake; moreover, and most of all, normal acid secretion acts as a physiologic stimulus promoting the intestinal secretion of secretin and CCK-PZ, as well as the subsequent stimulation of pancreatic exocrine secretion with better digestion of protein and fat (weight gain). Lastly, preservation of the stomach with resection of the pylorus favors better gastric emptying[28,29]. Regarding the impact of the reconstructive method on DGE, we think that the route of the gastro/duodeno-jejunal anastomosis with respect to the transverse colon (antecolic or retrocolic) or the type of reconstruction performed (Billroth I or Billroth II procedure) are not truly responsible for the differences in the prevalence of DGE after PD. We believe

Table 1 Summary of the cited studies on prevalence of delayed gastric emptying after pancreaticoduodenectomy

Ref.	Type of study	No. of patients	Studied groups	Significant difference in prevalence of DGE
Eshuis *et al*[22]	CCS	77	PD/PpPD + PJ-B Ⅱ AG/DJ	Not found
		77	PD/PpPD + PJ-B Ⅱ RG/DJ	
Oida *et al*[20]	CCS	14	MSSPPD + PG-B Ⅱ AGJ	PG-B Ⅱ RGJ < PG-B Ⅱ AGJ
		28	MSSPPD + PG-B Ⅱ RGJ	
Masui *et al*[26]	CCS	12	PpPD + PJ-B Ⅱ ADJ	PJ-B Ⅱ AMDJ < PJ-B Ⅱ ADJ
		106	PpPD + PJ-B Ⅱ AMDJ	
Kawai *et al*[17]	RCT	66	PrPD + PJ-B Ⅱ AGJ	PrPD < PpPD
		64	PpPD + PJ-B Ⅱ ADJ	
Kurahara *et al*[19]	RCT	22	SSPPD + PG-B Ⅰ RGJ	PG-B Ⅱ AGJ < PG-B Ⅰ RGJ
		24	SSPPD + PG-B Ⅱ AGJ	
Gangavatiker *et al*[24]	RCT	35	PD/PpPD + PJ-B Ⅱ AG/DJ	Not found
		37	PD/PpPD + PJ-B Ⅱ RG/DJ	
Chijiiwa *et al*[27]	RCT	17	PpPD + PJ-B Ⅱ ADJ	Not found
		18	PpPD + PJ-B Ⅱ VRDJ	
Tani *et al*[6]	RCT	40	PpPD + PJ-B Ⅱ ADJ	PJ-B Ⅱ ADJ < PJ-B Ⅱ RDJ
		40	PpPD + PJ-B Ⅱ RDJ	

CCS: Case control study; RCT: Randomized controlled trial; PD/PpPD: Pancreaticoduodenectomy or pylorus-preserving pancreaticoduodenectomy; PJ-B Ⅱ AG/DJ: Pancreaticojejunostomy with Billroth Ⅱ antecolic gastro/duodenojejunostomy; PJ-B Ⅱ RG/DJ: Pancreaticojejunostomy with Billroth Ⅱ retrocolic gastro/duodenojejunostomy; MSSPPD: Modified subtotal stomach-preserving pancreaticoduodenectomy; PG-B Ⅱ AGJ: Pancreaticogastrostomy with Billroth Ⅱ antecolic gastrojejunostomy; PG-B Ⅱ RGJ: Pancreaticogastrostomy with Billroth Ⅱ retrocolic gastrojejunostomy; PJ-B Ⅱ ADJ: Pancreaticojejunostomy with Billroth Ⅱ antecolic duodenojejunostomy; PJ-B Ⅱ AMDJ: Pancreaticojejunostomy with Billroth Ⅱ antecolic modified reconstruction with straightening of the stomach and twisted duodenojejunostomy; PrPD: Pylorus-resecting pancreaticoduodenectomy; PJ-B Ⅱ AGJ: Pancreaticojejunostomy with Billroth Ⅱ antecolic gastrojejunostomy; SSPPD: Subtotal stomach-preserving pancreaticoduodenectomy; PG-B Ⅰ RGJ: Pancreaticogastrostomy with Billroth Ⅰ retrocolic gastrojejunostomy; PJ-B Ⅱ VRDJ: Pancreaticojejunostomy with Billroth Ⅱ retrocolic modified reconstruction with vertical duodenojejunostomy; PJ-B Ⅱ RDJ: Pancreaticojejunostomy with Billroth Ⅱ retrocolic duodenojejunostomy; DGE: Delayed gastric emptying.

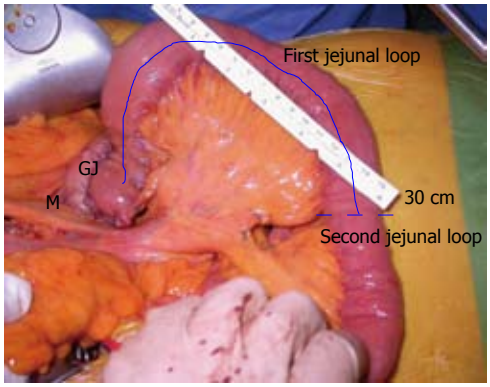

Figure 1 Retrocolic gastro-jejunal anastomosis in Roux-en-Y reconstruction after subtotal stomach-preserving pancreaticoduodenectomy. M: Mesocolic window; GJ: Gastro-jejunal anastomosis. Dashed line indicates the level of jejunal division.

that, after a PD, the impact of reconstructive methods on DGE is related mostly to the angulation or torsion of the reconstruction of the gastro/duodeno-jejunostomy because all the reported modified procedures associated with lower DGE, in Billroth I as well Billroth Ⅱ types of reconstruction, are related to the reconstructive anatomy of the alimentary circuit and are aimed to facilitate the outflow of the ingests from the gastric/duodenal remnant. An antecolic gastro/duodeno-jejunostomy can favor a straight construction and gastric emptying by gravity in a Billroth Ⅱ reconstruction after PD or PpPD[24] as well as a retrocolic Billroth Ⅱ gastrojejunostomy after a subtotal stomach-preserving pancreatoduodenectomy with pancreaticogastrostomy reconstruction can favor the transit of the gastric contents towards the jejunum in consequence of the retrogastric site of pancreaticogastric anastomosis[20,21]. A Billroth Ⅱ reconstruction can avoid the jejunal angulation produced by a Billroth I procedure in which the anastomosis of the proximal jejunum to the gastric/duodenal stump is performed at first, followed by pancreatico-jejunostomy and hepatico-jejunostomy[25] (or by hepatico-jejunostomy in a case in which a pancreatico-gastrostomy is carried out).

According to the ISGPS clinical criteria[16], we have recently reported a prevalence of 8.9% (8 cases) of grade A DGE and 1.1% (1 case) of grade C DGE in a series of 89 subtotal stomach-preserving PD followed by Roux-en-Y retrocolic reconstruction with anastomosis of the isolated Roux limb (i.e., first jejunal loop) to the stomach and single Roux limb (i.e., second jejunal loop) to the pancreatic stump and hepatic duct[30] (Figure 1).

We chose anastomosing the isolated proximal jejunum to the gastric remnant because, after removal of the duodenal source of CCK and secretin, preservation of the first jejunal loop in the reconstruction of the alimentary circuit maintains the physiologic jejunal secretion of secretin and CCK-PZ subsequent to alimentary transit and can compensate (at least in part) for the abolished duodenal hormonal release[29]. Then, the anastomosis of the isolated first jejunal loop to the gastric remnant, although retrocolic, avoided any angulation and torsion allowed the outflow of the gastric contents by gravity through a "straight route" (Figure 2). It is widely known that postoperative complications are related to the occurrence of DGE. Therefore, controlling the prevalence

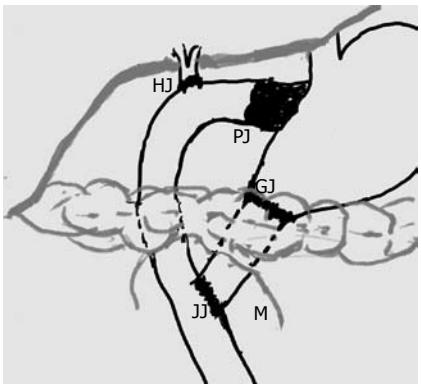

Figure 2 Roux-en-Y retrocolic reconstruction. HJ: Hepatico-jejunal anastomosis; PJ: Pancreatico-jejunal anastomosis; GJ: Gastro-jejunal anastomosis; JJ: Jejuno-jejunal anastomosis; M: Mesocolic window.

of other postoperative complications can contribute to reduce the occurrence of DGE. Postoperative pancreatic fistula occurred in seven patients (7.8%) of our series. Six cases of grade A fistula resolved spontaneously and in only one grade B fistula was percutaneous drainage necessary. Postoperative hemorrhage occurred in two of 89 (2.2%) patients, biliary fistula in eight (8.9%) patients and acute pancreatitis in one (1.1%). One patient with pre-existing stenosis of the hepatic artery developed thrombosis of the hepatic artery.

In conclusion, PrPD may contribute to a decrease in the prevalence of DGE due to pylorospasm, denervation and devascularization of the pylorus ring, which may occur after PpPD. A "straight" route, not necessarily an "antecolic" route, may obviate to the prevalence of DGE due to torsion or angulation in the reconstruction of the alimentary tract.

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S- Editor Song XX **L- Editor** A **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v18.i45.6532

World J Gastroenterol 2012 December 7; 18(45): 6532-6536
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

FIELD OF VISION

Tumor budding as a potential histopathological biomarker in colorectal cancer: Hype or hope?

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Author contributions: Grizzi F conceived and wrote this manuscript; Celesti G and Basso G discussed the topic; Laghi L discussed the topic and revised this manuscript.
Supported by Ministero dell'Istruzione, dell'Università e della Ricerca, Target Project Oncologia 2006; Alleanza Contro il Cancro; the Italian Association for Cancer Research, grant project No. IG5256
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Received: September 19, 2012 Revised: October 12, 2012
Accepted: November 11, 2012
Published online: December 7, 2012

Abstract

Colorectal cancer (CRC), the third most commonly diagnosed type of cancer in men and women worldwide is recognized as a complex multi-pathway disease, an observation sustained by the fact that histologically identical tumors may have different outcome, including various response to therapy. Therefore, particularly in early and intermediate stage (stages Ⅱ and Ⅲ, respectively) CRC, there is a compelling need for biomarkers helpful of selecting patients with aggressive disease that might benefit from adjuvant and targeted therapy. Histopathological examination shows that likely other solid tumors the development and progression of human CRC is not only determined by genetically abnormal cells, but also by intricate interactions between malignant cells and the surrounding microenvironment. This has led to reconsider the features of tumor microenvironment as potential predictive and prognostic biomarkers. Among the histopathological biomarkers, tumor budding (i.e., the presence of individual cells and small clusters of tumor cells at the tumor invasive front) has received much recent attention, particularly in the setting of CRC. Although its acceptance as a reportable factor has been held back by a lack of uniformity with respect to qualitative and quantitative aspects, tumor budding is now considered as an independent adverse prognostic factor in CRC that may allow for stratification of patients into risk categories more meaningful than those defined by tumor-node-metastasis staging alone, and also potentially guide treatment decisions, especially in T2-T3 N0 (stage Ⅱ) CRCs.

Key words: Colorectal cancer; Tumor budding; Biomarker; Histopathology

Peer reviewer: Asmaa Gaber Abdou, Assistant Professor, Department of Pathology, Faculty of Medicine, Menofiya University, Shebein Elkom 002-048, Egypt

Grizzi F, Celesti G, Basso G, Laghi L. Tumor budding as a potential histopathological biomarker in colorectal cancer: Hype or hope? *World J Gastroenterol* 2012; 18(45): 6532-6536 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i45/6532.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i45.6532

INVITED COMMENTARY ON HOT ARTICLES

We read with great interest the recent article by Lugli *et al*[1] describing the morphology of "tumor budding" as a promising histopathological prognostic feature in colorectal cancer (CRC) and strongly recommend it to the readers.

Although in certain countries a decline in CRC incidence rate has been registered, attributed to increases in screening adhesion rates and linked detection and removal of precancerous polyps[2], CRC remains one of the most

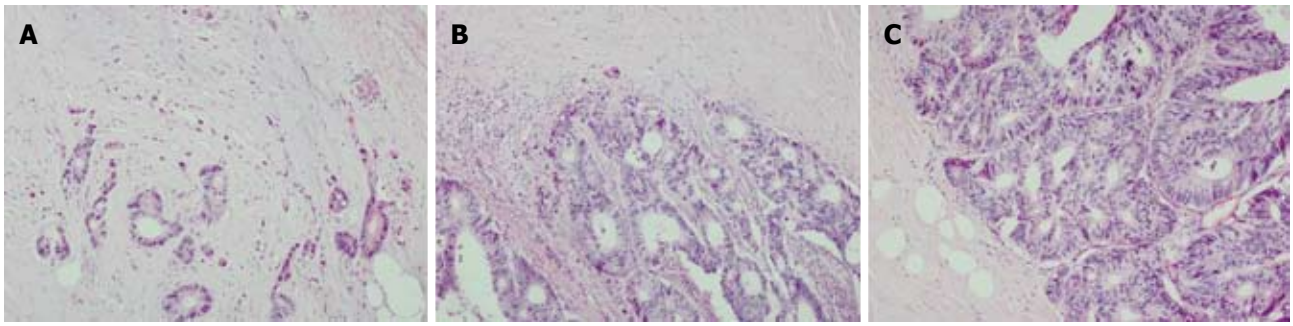

Figure 1 Colorectal cancer at the invasive front shows different growth patterns. Tumor budding denotes the presence of isolated single neoplastic cells or small clusters of cells scattered in the stromal compartment at the invasive tumor margin (A), although intra-tumoral budding is also reported. Tumor margin organized in larger tumor cell clusters (B) and a smooth infiltration tumor margin (C). Hematoxylin and eosin stain, objective magnification 20×.

common cancers[3]. By its frequency, CRC ranks third in men and women worldwide[3]. Explained as a multi-step dynamical disease in the last two decades, CRC develops slowly over several years and progresses through cytologically distinct benign and malignant states, from single crypt lesions through adenoma, to malignant carcinoma with the potential for local invasion and distant metastasis[4,5]. According to the model of multi-step carcinogenesis, adenomatous cells accumulate a number of molecular abnormalities to eventually become fully malignant[6,7]. In spite of unifying theories, genetic and epigenetic events during the carcinogenesis process differ considerably from tumor to tumor. Thus, CRC is not a unique disease; rather it encompasses different molecular and pathological entities with a wide range of clinical behaviors[8]. At the molecular level, CRC encloses a complex array of gene alterations. Essentially, like individual fingerprints, each tumor arises and behaves in a distinctive fashion that is unlikely to be fully recapitulated by any other tumor. Nevertheless, molecular changes allow for a basic categorization of CRC, which is largely acknowledged, although likely over-simplistic. It has been demonstrated that genetic and epigenetic features, such as microsatellite instability (MSI), chromosomal instability, CpG island methylator phenotype or even global DNA hypomethylation, lead to alterations of gene function on a genome-wide scale. It is known that activation of oncogenes, including *KRAS*, *BRAF*, *TGFBR*, *PIK3CA* and *TP53*, affects complex intracellular signaling pathways[9,10]. The suppressor pathway is disrupted in CRC with chromosomal instability occurring in the majority of CRCs (nearly, 85%), which have a molecular profile characterized by specific chromosomal amplifications and transformations, aneuploidy, and loss of heterozygosity[8-10]. Differently, CRCs of the mutator pathway (roughly, 15%) have a defective DNA mismatch repair (MMR) system, which leads to accumulation of unrepaired mutations[9], and harbor frameshift mutations in coding mononucleotide repeats of cancer-related genes (targets)[11]. It is now accepted that MSI CRCs have a heterogeneous histological appearance, better prognosis due to a reduced metastatic potential, and a different response to 5-fluoro-uracyl[12-14].

Histopathology of CRC

Histopathological examination shows that likely other solid tumors, CRCs are infiltrated by various innate and adaptive immune cells[15-17], and that in the cancer context, epithelial cells coexist with different extracellular matrix components and non-neoplastic cell types, including fibroblasts, myofibroblasts, adipocytes, endothelial cells, pericytes, which collectively form the tumor microenvironment[18].

It is well known that histopathology reports usually include various features and including tumor grade, histological sub-type, state of resection margins and information on vascular and perineural invasion, but the tumor-border configuration (i.e., growth pattern) and especially tumor budding remain rarely described[1]. The term "tumor budding" denotes the presence of isolated single neoplastic cells or small clusters of cells (conventionally, up to 5 cells) scattered in the stromal compartment at the tumor invasive margin (Figure 1)[19-21].

Tumor stage as stated by the American Joint Committee on Cancer/International Union Against Cancer (AJCC/UICC) tumor-node-metastasis (TNM) system is currently considered as the most robust prognostic criterion for CRC patients. The inability of the AJCC/UICC staging system to accurately predict the outcome of individual patients with stage Ⅱ and stage Ⅲ CRC might be overcome by adding morphological, molecular or treatment-related features, that could stratify patients more accurately into different risk categories[22]. Depth local tumor infiltration (pT), loco-regional lymph-node involvement (N status), venous and lymphatic invasion, and tumor grade, are currently recognized as the main histopathological characteristics associated with worse patient outcome.

Tumor budding and CRC

Tumor budding first introduced by Jass *et al*[23], as a reliable histopathological hallmark to estimate the aggressiveness of rectal cancer, was initially shown to have a superior prognostic value when compared to other histopathological characteristics, including tumor differentiation and venous invasion. Tumor grading based on the nature of the advancing tumor margins, which in the scoring sys-

tem proposed by Giger *et al*[24] divided rectal tumors into expanding type and infiltrating type, obtained wide acceptance among surgical pathologists worldwide. Although subsequent studies revealed a scarce reproducibility of Jass scoring system, several authors highlighted the potential role of tumor budding as a valid prognosticator also in tumors other than CRC, including lung cancer[25,26], invasive ampullary adenocarcinomas[27], and oesophageal and gastro-oesophageal junction cancers[28]. In CRC, tumor budding is considered as a stage ⅡB prognostic factor, and strictly associated with lymph-node metastasis[1]. It has been shown that the presence of "buds" at the tumor invasive front represents an independent predictor of lymph node metastasis in patients with sub-mucosal invasive or early pT1 CRCs[29]. It has also been suggested that the frequency of tumor budding increases with more advanced TNM stage[1].

Tumor budding is virtually absent in MMR-deficient cancers[30,31]. MSI CRCs have significantly more pronounced tumor infiltrating lymphocytes (i.e., $CD3^+$ or $CD8^+$ cells), peritumoral lymphocytes inflammation, and bundling edge (i.e., the ability of cells to adhere and to migrate) compared with microsatellite-stable CRCs, all factors contributing to the absence of tumor budding in MSI CRCs.

Tumor budding and the epithelial-mesenchymal transition

A parallel between tumor budding and the epithelial to mesenchymal transition (EMT) has also been recently proposed. This (potentially reversible) process thought to occur physiologically during embryological development (EMT subtype Ⅰ), has been also associated with wound healing, tissue regeneration, organ fibrosis (EMT subtype Ⅱ), and tumor invasion (EMT subtype Ⅲ). Cells in EMT loss their epithelial phenotype (i.e., lack of E-Cadherin and cell polarity, expression of transcription factors including the zinc finger proteins SNAIL and SLUG, TWIST, ZEB 1/2 and SMAD) and dynamically acquire a mesenchymal phenotype (i.e., taking on a spindle-like, fusiform morphology, become motile, and start expressing mesenchymal markers including N-cadherin, fibronectin and vimentin)[32]. While the mechanisms promoting distant metastasis are extremely wide and still under intense investigation, the presence of EMT features in cells of the tumor microenvironment has been associated with an increased metastatic potential[32,33].

Assessing the tumor budding in colorectal cancer tissues

Rapidly growing insights into the cell biology of CRC and the recent developments of high-throughput technologies, gene sequencing and molecular diagnostics have led to practicable expectations for the identification of molecular biomarkers to be used in optimized and tailored treatment regiments. However, histopathological interpretation of CRC tissues remains the gold standard for cancer diagnosis. Tissue specimens, consisting of different cell types related to each other in complex spatial patterns, are important resources for both primary research efforts and validation of biological findings that are made in laboratory[34]. Working with human tissues poses several challenges to investigators, including: (1) tissue sampling (i.e., appropriate processing, histological variability, tissue heterogeneity with different areas of cancer, necrosis, inflammation and natural tissue); (2) selection of the proper preservation technique (i.e., maintenance of tissue morphology and molecular profile); (3) tissue complexity (i.e., requirement of an accurate histopathological interpretation); and (4) not least ethical and legal rules. However, an approach that integrates histopathology and molecular biology within a unique translational system is a mandatory strategy to pursue a better understanding of cancer. Such an effort can be achieved only through a more effective incorporation of pathology into clinical research, and conversely by integrating biological research into the pathological assessment, likely through efficient networks of translational researchers joining their data.

The morphology of the tumor invasive front has come into the focus of scientific studies because it appears to be intimately linked to cancer aggressiveness. Despite the established prognostic relevance of tumor budding in CRC, the reproducibility of actual methods proposed for its assessment, however, remains unstandardized, limiting its application in routine pathology practice[35]. Diagnostic reproducibility is a prerequisite for the validation of a diagnostic test and is crucial for patient care. Tumor budding promises to be a histopathological prognostic factor in CRC, and although the level of agreement needs to be improved and further investigations are compulsory to confirm any association between the rates of tumor budding detection and clinical outcome, its evaluation can be improved first by an appropriate physician training. In addition, the use of immunohistochemistry (IHC) highlights budding cells by pan-cytokeratin antibodies leading to a significant increase of tumor budding-positive cases. Single tumor cells can be more accurately detected by immunological techniques than standard hematoxylin and eosin staining, even when they appear at the tumor boundary showing glandular disruption. Under these circumstances, dissociated tumor cells should not be interpreted as budding to avoid biasing tumor budding evaluation[31].

As tumor budding has been shown as an independent prognostic factor in CRC, particularly in node-negative disease, its assessment has the potential to increase prognostic accuracy and influence treatment algorithms. When examined carefully, the majority of CRCs display some degree of budding; hence, attempts have been made at developing scoring systems to identify a prognostically significant degree of budding, commonly termed "high-grade" budding. Definitions of high-grade budding, however, vary substantially among different observers and even among different studies by the same observers[31].

Final remarks

Consensus criteria for its evaluation must be better estab-

lished, to guide further research in this area and to provide the practicing pathologist with reporting guidelines. With respect to setting these criteria, studies focusing on budding should be designed to define objective cut-off for meaningful tumor budding. In mathematical terms, also tumor budding is a continuous variable; thus, a cut-off threshold should be less arbitrary as possible, and an attempt should be made to identify the budding threshold that results into relevant predictive information. Along this line, pathologist reporting on tumor budding should provide detailed information regarding the qualitative and quantitative criteria used to evaluate budding in order to allow for meaningful comparisons among different studies. Finally, the role of IHC in the evaluation of budding needs to be clarified. Although it might be impractical to perform IHC on all CRCs, there may be certain cases (i.e., in the context of a remarkable inflammatory reaction at the tumor invasive front), where it may reveal buds that are dubious when observed in standard hematoxylin and eosin stained histological sections.

It is indubitable that the substantial impediment to the adoption of tumor budding as a routinely reportable feature is the lack of a well defined, standardized and quantitative assessment. At any event, due to the forceful evidence that tumor budding is one of the most promising prognostic factors actually available, it is incumbent on the scientific community participating to the identification of CRC prognostic factors to move promptly to addressing it and removing the obstacles to its routine reporting and comparison with other predictive factors.

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S- Editor Song XX **L- Editor** A **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v18.i45.6537

World J Gastroenterol 2012 December 7; 18(45): 6537-6540
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Anti-tumor immunity, autophagy and chemotherapy

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Received: August 18, 2012 Revised: October 25, 2012
Accepted: November 6, 2012
Published online: December 7, 2012

Abstract

Autophagy or self-digestion of cells is activated upon various stressful stimuli and has been found to be a survival and drug resistance pathway in cancer. However, genetic studies support that autophagy can act as a tumor suppressor. Furthermore, defective autophagy is implicated in tumorigenesis, as well. The precise impact of autophagy on malignant transformation has not yet been clarified, but recent data suggest that this complex process is mainly directed by cell types, phases, genetic background and microenvironment. Relation of autophagy to anticancer immune responses may indicate a novel aspect in cancer chemotherapy.

Key words: Cancer; Autophagy; Chemotherapy; Antitumor immunity; Cell death

Peer reviewers: Hubert Blum, Professor, University of Freiburg, Hugastetter Strasse 55, L-79106 Freiburg, Germany; Assy Nimer, MD, Assistant Professor, Liver Unit, Ziv Medical Centre, BOX 1008, Safed 13100, Israel

Műzes G, Sipos F. Anti-tumor immunity, autophagy and chemotherapy. *World J Gastroenterol* 2012; 18(45): 6537-6540 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i45/6537.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i45.6537

INVITED COMMENTARY ON HOT ARTICLES

Cancer is one of the major health problems worldwide, therefore constantly more effective therapeutic strategies are expected. Cancers arise from the uncontrolled proliferation and spreading of malignantly transformed cell clones with the obvious ability to evade protective immunity. In view of immune surveillance selective, specific and effective eradication of various cancer cells by a subsequent active host immune response serving as a widespread therapy option has still been remained unsolved.

Current therapies for cancer mainly are based on chemotherapeutic drugs that kill transformed, dividing cells or block cell division, but unfortunately these treatments may also attack normal proliferating cells, including immunocompetent ones. However, targeted immune responses (immunotherapy) to tumors may be specific, thus making the possibility to avoid normal cell injury. According to therapeutic vaccines killed tumor cells or tumor antigens can efficiently induce anticancer immunity.

So far less attention has been paid on the possible subcellular and molecular impact of chemotherapy-induced cell death regarding induction of host immune responses.

In a recent experimental study Michaud *et al*[1] have underscored a new aspect of anticancer chemotherapy, that autophagy may contribute to action of certain drugs eliciting immunogenic tumor cell death. This type of cellular fate is characterized biochemically by pre- and postapoptotic events, like calreticulin exposure and high mobility group B-1 (HMGB-1) secretion, and by ATP release.

Autophagy

Besides the proteasomal degradation pathway autophagy represents an additional evolutionarily highly conserved multi-step process of cellular self-digestion due to sequestration of excessive, damaged, or aged proteins and

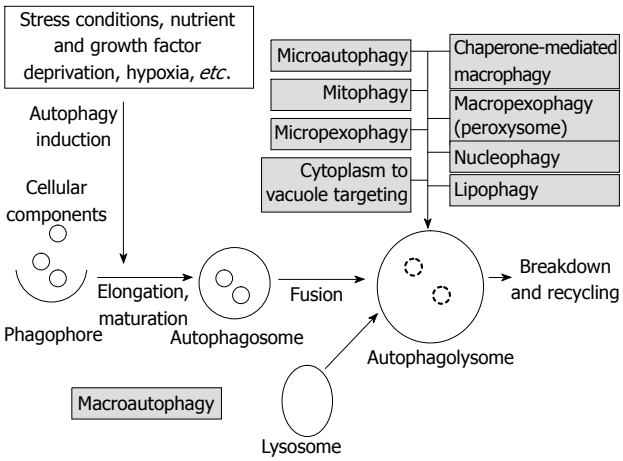

Figure 1 The process of macroautophagy and the types of autophagy (gray boxes).

intracellular organelles in double-membranous vesicles of autophagosomes, terminally self-digested in lysosomes[2].

Different types of autophagy according to the route of delivery to lysosomes and the main physiological functions have been characterized, like macro- and microautophagy, and chaperon-mediated autophagy[3]. Upon specific targeted degradation of cytosolic proteins, lipids, or organelles (e.g., ribosomes, nucleosomes, mitochondria), selective forms of autophagy can further be classified as lipophagy, or ribophagy, nucleophagy and mitophagy[2].

Macroautophagy (hereafter simply termed autophagy) refers to cytoplasmatic bulk, non-selective degradation of subcellular constituents. Within this complex catabolic pathway regulated tightly by a limited number of autophagy genes (atgs) various morphologic stages are distinguishable starting with the formation of phagophore, followed by its elongation and maturation to autophagosome, and finally the fusion with lysosomes[4]. The process of macroautophagy and the types of autophagy are summarized in Figure 1.

Autophagy is deeply implicated in regulation of numerous physiologic functions including cell development and differentiation, survival and senescence, and it also crucially affects inflammation and innate and adaptive immunity[5]. On a basal level intact autophagy serves constantly and constitutively as a critical adaptive and surveillance mechanism in maintaining cellular homeostasis[3]. However, autophagy is inducible, as well in response to different cellular metabolic stress conditions, including nutrient and growth factor deprivation in order to preserve cell viability. Defects in basal autophagy may yield accumulation of cytotoxic materials, damaged DNA, and thus, genomic instability, while alterations of induced autophagy especially lead to reduced cell survival[4,5].

In general, defective autophagy by compromising cellular fitness has been ultimately related to several disease conditions, such as cancer, certain neurodegenerative, liver, and infectious disorders, aging, and inflammatory conditions, like Crohn's disease[3,5-7].

Regarding tumorigenesis a dual-faced (Janus) role of autophagy has been proposed, since on one side it may be critical for cancer cell survival and progression, in particular under stressful situations, however it may elicit tumor death signaling pathways. Direction of autophagy toward cytoprotection or tumor cell suppression, thus the pro-survival or pro-death function is context-dependent, and influenced by many intra- and extracellular factors, such as involved tissues, surrounding microenvironment, genetic background, and stages of tumor development, nevertheless its precise relation to cancer networks has not yet been fully elucidated[5,6,8].

The involvement of autophagy in cell death, either in apoptosis (programmed, type I death) or in non-apoptotic or necrotic death, and their possible interactions are rather complicated. Autophagy in tumor cells usually displays a critical, programmed pro-survival function by inhibiting apoptosis or suppressing necrotic death, including programmed (or regulated) cell necrosis of caspase-independent necroptosis, and poly-ADP-ribose polymerase-mediated necrosis[9].

In cases of autophagy deficiency, however, no tumor suppression, but on the contrary, accelerated tumorigenesis can be manifestated. In autophagy-incompetent cells upon induced oxidative stress cell-autonomous mechanisms are exhibited in forms of accumulated DNA damage and chromatin instability[10]. As a non-cell-autonomous mechanism, however, inflammatory events along with defective apoptosis could also contribute independently to cancer progression, partly by favouring cell necrosis[11]. Similar situation has been found in human inflammatory bowel diseases (IBD) with high risk of malignancy, and in experimental cases of atg5-/- or atg7-/- mice displaying inflammatory Paneth cell abnormalities resembling human IBD[7,12].

The *atg6/Beclin-1* gene, a Bcl-2/Bcl-xL interacting element has been found to be monoallelically lost in certain human cancers, and confirmed that it functions as a haploinsufficient tumor suppressor[13]. However, this suppressive function of Beclin-1 may be tissue-specific, since even its higher expression has been detected in colorectal and gastric carcinomas[14]. In addition to Beclin-1, alterations of other autophagy-associated genes, e.g., atg4, atg5, UV-irradiation resistance-associated gene (UVRAG), or Bax-binding protein-1 (Bif-1) have also been detected in various cancers, indicating that tumor suppression is attributed to different autophagy elements. Nonsense mutations of UVRAG, and downregulation of Bif-1 have been documented in colon and gastric carcinomas, and in colon adenocarcinomas, respectively[15-17].

Hypothetically, increased autophagic flux *via* excessively induced autophagy may promote non-apoptotic (programmed, type Ⅱ) autophagic cell death, acting like a tumor suppressor[18]. Autophagy is also known to stimulate oncogene-induced senescense, thus providing another possible barrier against malignant transformation[19]. Nevertheless, there is no direct evidence regarding the realistic anti-tumor capacity of autophagy.

In human cancers constitutive activation of Ras- and phosphoinositol 3-kinase/Akt-mammalian target of rapa-

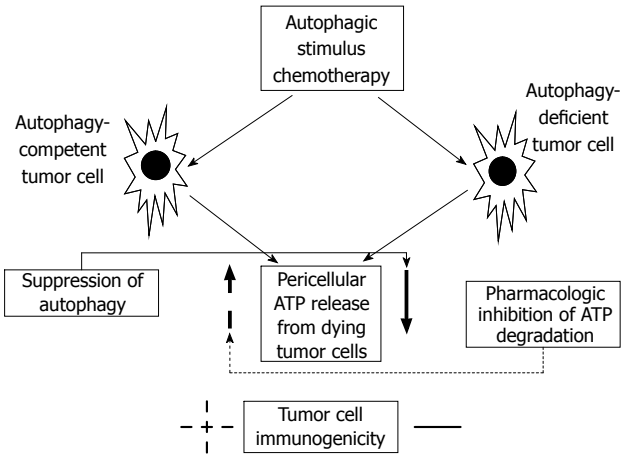

Figure 2 The relation of autophagy and anticancer immunity. Similarly to autophagy-deficient tumor cells inhibition of autophagy results in decreased pericellular ATP secretion, and thus suppressing anticancer immunity. Pharmacologic inhibition of ATP degradation, however, increases ATP level in the microenvironment of tumor cells, and favours tumor cell immunogenicity.

mycin (mTOR) pathway is a common phenomenon, and mTOR complex 1 seems to be the main negative regulator of autophagy[20,21]. The tumor suppressor p53 gene exerts a typical dual role in autophagy regulation, depending primarily on its subcellular, nuclear or cytoplasmic distribution[22]. Both stress-responsive cellular degradation pathways of intrinsic and extrinsic apoptosis and of autophagy can fundamentally affect, activate or inhibit each other *via* an extensive molecular crosstalk, and in fact, cell destiny is determined by their actual functional status and interplay[6,23]. Their crosstalk is regulated primarily by the current status of the Bcl-2/Beclin-1 complex, dissociation of which can be achieved upon activation of mitogen activated phosphokinase-jun kinase or translocation of the damage-associated molecular pattern (DAMP) protein HMGB-1[23]. Nuclear factor (NF)-κB plays also a critical role in malignant transformation, and its constitutive, chronic activation has been observed in the majority of different tumor cells. There is also a complex interaction between autophagy and the NF-κB signaling pathways *via* positive and negative feedback regulatory loops[24]. The important autophagy selective substrate p62 acts as an adaptor protein to regulate NF-κB, as well[25].

Overall, there is no doubt that process of autophagy can be considered as an apparently quite difficult regulatory network, being in close connection with other signal transduction pathways and cellular programs. The complex and rather contradictory function of autophagy in tumorigenesis makes itself a promising but challenging therapeutic target both in cancer treatment and prevention. In autophagy-competent tumor cells autophagy increase can often be induced in response to different chemo- and radiotherapies, representing mainly an adaptive survival mechanism, but provoking simultaneously treatment resistance. Therefore it has been hypothesized that concurrent pharmacologic inhibition of autophagy, as an adjuvant may sensitize tumor cells to a spectrum of anticancer drugs[22,26,27]. In cases of autophagy-deficient tumors, however, due to their extreme susceptibility, metabolic stress- and DNA-damage-inducing therapeutic protocols are suggested. However, autophagy induction could also provide an alternative therapeutic option[22,26,27]. Nevertheless, excessive autophagy can potentially act as an active cell death machinery, mainly along with inherent apopotosis defects, so induction of autophagy by antitumor drugs may also be considered as an efficient cytotoxic manipulation.

Michaud *et al*[1] in their experiments, using transplantable murine tumors of CT26 colorectal carcinoma and of MCA205 fibrosarcoma treated either with mitoxantrone or oxalipatin have found that autophagy-competent tumor cells release more ATP comparing with autophagy-deficient ones. Furthermore, pharmacologic inhibition of autophagy reduced chemotherapy-induced ATP release, however induction of autophagy did not trigger it. ATP serves as a danger signal, it is a prominent DAMP molecule. In addition, unlike autophagy-deficient tumor cells chemotherapy in autophagy-competent cancer cells elicited a protective immune response, i.e., attraction of dendritic cells, CD4+ and CD8+ lymphocytes, and priming of T cells. Inhibition of autophagy decreased the immunogenic potential of tumor cells. The authors finally conclude, that upon chemotherapy premortem autophagy is required for tumor immunogenicity by releasing ATP from dying apoptotic cells, and consequently, in case of autophagy defiency the ability of tumor cells to induce an adaptive anticancer immune response is significantly restricted. In that transplantable model dying cancer cells function as a therapeutic vaccine. Nevertheless, in autophagy-deficient tumors of immunocompetent hosts by pharmacologic inhibition of ATP degradation a compensatory increase in pericellular ATP content was achieved, thus successfully restoring the immunogenic capacity, and suggesting a novel adjuvant therapeutic possibility (Figure 2).

Findings of Michaud *et al*[1] not only highlight on the complexity and many faces of autophagy in tumorigenesis, but emphasize the rationality of analyzing subcellular, molecular consequences of chemotherapy in respect of influencing host immunity, and thus propose a promising therapeutic strategy to compensate autophagy deficiency-related altered tumor immunogenicity.

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S- Editor Huang XZ **L- Editor** A **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v18.i45.6541

World J Gastroenterol 2012 December 7; 18(45): 6541-6545
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

FIELD OF VISION

Solitary rectal ulcer syndrome in children: A literature review

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Received: August 2, 2012 Revised: September 20, 2012
Accepted: September 29, 2012
Published online: December 7, 2012

Abstract

Solitary rectal ulcer syndrome (SRUS) is a benign and chronic disorder well known in young adults and less in children. It is often related to prolonged excessive straining or abnormal defecation and clinically presents as rectal bleeding, copious mucus discharge, feeling of incomplete defecation, and rarely rectal prolapse. SRUS is diagnosed based on clinical symptoms and endoscopic and histological findings. The current treatments are suboptimal, and despite correct diagnosis, outcomes can be unsatisfactory. Some treatment protocols for SRUS include conservative management such as family reassurance, regulation of toilet habits, avoidance of straining, encouragement of a high-fiber diet, topical treatments with salicylate, sulfasalazine, steroids and sucralfate, and surgery. In children, SRUS is relatively uncommon but troublesome and easily misdiagnosed with other common diseases, however, it is being reported more than in the past. This condition in children is benign; however, morbidity is an important problem as reflected by persistence of symptoms, especially rectal bleeding. In this review, we discuss current diagnosis and treatment for SRUS.

Key words: Solitary rectal ulcer syndrome; Rectal bleeding; Children; Diagnosis; Treatment

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Dehghani SM, Malekpour A, Haghighat M. Solitary rectal ulcer syndrome in children: A literature review. *World J Gastroenterol* 2012; 18(45): 6541-6545 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i45/6541.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i45.6541

INVITED COMMENTARY ON HOT ARTICLES

Solitary rectal ulcer syndrome (SRUS) is an uncommon chronic and benign rectal disorder often related to abnormal defecation or straining. It was first described by Cruveilhier[1] in 1829, when he reported four unusual cases of rectal ulcers. The term "solitary ulcers of the rectum" was used by Lloyd-Davis in the late 1930s and in 1969 the disease became widely recognized after a review of 68 cases by Madigan *et al*[2] and few years later, a more detail pathogenic concept of the disease was reported by Rutter *et al*[3]. SRUS is an infrequent or unrecognized or misdiagnosed disorder, with an estimated prevalence of 1 in 100 000 persons per year[4]. Solitary rectal ulcer is a misnomer as ulcers are found in 40% of patients, while 20% of patients have a solitary ulcer, and the rest of the lesions are different in shape and size, including hyperemic mucosa to broad-based polypoid lesions[5]. The disease process also may involve the sig-

moid colon[6].

Although it is uncommon, it is well recognized in adult populations[3]. SRUS seems to be rare in childhood[7-10] and may masquerade as other more common conditions, causing difficult-to-manage lower gastrointestinal symptoms. Opinion differs regarding the best treatment for this troublesome condition, varying from conservative management and enema preparations to more invasive surgical procedures such as rectopexy[11].

In this review article, several aspects of this syndrome will be evaluated with an especial focus on the condition in children. Detailed risk factors, causes, and treatment methods will help guide future treatment and prevention strategies in children.

Pathophysiology and clinical presentation

The pathophysiology of SRUS is incompletely understood; however, rectal hypersensitivity leading to the persistent desire to defecate and sensation of incomplete evacuation may have a role in SRUS[12]. Inappropriate contraction of the puborectalis muscle and rectal mucosal prolapse have been commonly implicated, although trauma and ischemia have been suspected in some children[7,13]. In children, secondary to chronic mechanical and ischemic trauma, inflammation by hard stools, and intussusception of the rectal mucosa, some histological features of SRUS can be seen such as fibromuscular obliteration of the lamina propria and disorientation of muscle fibers[14].

In our previous study of 256 children who were evaluated endoscopically for recurrent lower gastrointestinal bleeding, 4.7% had this syndrome[15]. In adult patients, men and women are affected equally, with a small predominance for women[16], but 75%-80% of children with SRUS are boys[15,17]. Suresh *et al*[18] have evaluated 325 children aged < 18 years during 8 years for various indications such as bleeding, polyps and anal fissure. Twenty-two (6.8%) children were diagnosed with SRUS and ranged in age from 18 months to 18 years (median: 10 years), and 18 (81.8%) of these were ≥ 8 years of age. The male to female ratio in this group was 1.4:1. To date, the youngest patient with SRUS was a child of 1.5 years[18]; Gabra *et al*[19] also have reported two boys with SRUS who were 2 and 3 years old.

The average time from the onset of symptoms to diagnosis is 5 years, ranging from 3 mo to 30 years in adults, which is longer than in pediatric patients (3.2 years, range: 1.2-5.5 years)[15,17,20-22]. This syndrome results from obstructed defecation secondary to internal rectal prolapse with a collection of symptoms including rectal bleeding, passage of mucus and straining on defecation, perineal and abdominal pain, tenesmus, feelings of incomplete defecation, constipation, and rectal prolapse[23-25]. The amount of blood varies from a little fresh blood to severe hemorrhage that requires blood transfusion[26-28]. Up to 26% of patients can be asymptomatic and may not show the bleeding that is discovered incidentally while investigating other diseases[5]. The use of digital manipulation to assist with a bowel movement is variably reported in patients with SRUS[15,29]. Bright-red blood from the rectum or mucoid rectal discharge, tenesmus, proctalgia, and constipation are the major symptoms. Some children present with apparent diarrhea (because of prolonged visits to the bathroom) and the associated bleeding, abdominal pain, and tenesmus may suggest to clinicians the presence of inflammatory bowel disease[25].

Diagnosis

SRUS is a relatively uncommon but bothersome and easily misdiagnosed condition of childhood. Clinical suspicion and paraclinical evaluations are needed and diagnosis is via symptomatology in combination with endoscopic and histological findings[17]. A complete and thorough history is most important in the initial diagnosis of SRUS. It is essential to differentiate SRUS from other devastating, chronic, and potentially lethal disorders such as inflammatory bowel disease, amebiasis, lymphogranuloma venereum, chronic ischemic colitis, endometriosis, colitis cystica profunda, and malignancy. Obstructive symptoms (anismus) in children may be interpreted by parents as constipation. Concomitant haematochezia may be misinterpreted as originating from an anal fissure caused by constipation, or as other causes of rectal bleeding such as a juvenile polyp[30-32].

Defecography is a useful method for determining the presence of intussusception or internal or external mucosal prolapse and can demonstrate a hidden prolapse, as well as a non-relaxing puborectalis muscle and incomplete or delayed rectal emptying[33]. Barium enema shows granularity of the mucosa, polypoid lesion, rectal stricture and ulceration, and thickened rectal folds; all of which are nonspecific findings[33,34]. Temiz *et al*[35] have recommended that defecography and anorectal manometry should be performed in all children with SRUS to define the primary pathophysiological abnormality and to select the most appropriate treatment protocol.

The endoscopic spectrum of SRUS varies from simple hyperemic mucosa to small or giant ulcers to broad-based polypoid lesions of different sizes. Macroscopically, SRUS typically appears as shallow ulcerating lesions on a hyperemic surrounding mucosa, most often located on the anterior wall of the rectum at 5-10 cm from the anal verge. Ulcers may range from 0.5 to 4 cm in diameter but usually are 1-1.5 cm in diameter[5,15,30].

Histological examination of biopsy material is necessary to confirm a diagnosis of SRUS. The histological criteria for diagnosis are as follows: fibrous obliteration of the lamina propria, streaming of fibroblasts and muscle fibers between crypts, thickening of muscularis mucosa, branching and distorted glandular crypts and diffuse collagen infiltration of the lamina propria[15,36-38].

Recent studies have shown the usefulness of anorectal ultrasound in assessing internal anal sphincter thickness, which is shown to be increased in patients with this syndrome[37,39], and it has been suggested that

sonographic evidence of a thick internal anal sphincter is highly predictive of high-grade rectal prolapse and intussusception in patients with SRUS[39].

There is a need for a high index of suspicion for the possibility of SRUS in young children with clinical picture of nonspecific proctitis.

Treatment

The most frustrating aspect of SRUS is the difficulty in treatment; experiences have shown that most therapeutic regimens are inadequate. There are few data on treatment and its outcome in children with SRUS. In most reported pediatric case series, active intervention using enemas[15], laxatives[40], and surgical approaches have been used more frequently than behavioral modification, mainly biofeedback therapy in adults[11,41-45].

Some suggestions for the treatment of SRUS include reassurance of the patient and parents that the lesion is benign, encouragement of a high-fiber diet[46], avoidance of straining, regulation of toilet habits, and attempt to discuss any psychosocial factors[20,36,37,47,48]. The use of a high-fiber diet, in combination with stool softeners and bulking laxatives, and avoidance of straining have had varying responses[4,46].

In children, primary medical treatment is proposed for most cases[49]. Topical application of sucralfate can be effective for treatment of SRUS in some patients[15,50]. Many medications that are useful in the treatment of patients with inflammatory bowel disease have been tried in those with SRUS, such as sulfasalazine and corticosteroids, with varying responses[16,51]. In one study, oral salicylate and other topical agents such as mesalamine and steroids were not effective[52]. Endoscopic application of human fibrin sealant[53], laser therapy[54], and biofeedback[45,47,48] are some of the effective treatment methods for SRUS.

A therapeutic role for botulinum toxin injection into the external anal sphincter for the treatment of SRUS and constipation associated with dyssynergia of defecation dynamics has been reported by Keshtgar *et al*[55]. The effect of botulinum toxin lasts approximately 3 mo, which may be more beneficial than biofeedback therapy[55].

Surgical methods for treatment of SRUS are rectopexy[42,43,56], excision[4,5,16] and Delorme's procedure[41,44,57,58].

The choice of treatment protocol depends on acuteness of symptoms and whether there is an underlying rectal prolapse or not[25,31]. Maintaining compliance in children may prevent progression to the type of long-term morbidity and treatment resistance sometimes seen in adults with this condition[25]. Recommended treatment in children by Abbas *et al*[59] is initially conservative, but, if that fails, transrectal resection followed by a high-fiber diet. Conservative management including behavioral modification and reduction of time spent straining at defecation has been reported as a good method[25,46,60].

Compliance with simple behavioral modification appears to produce a good outcome in childhood SRUS, probably because of the short disease duration compared with adults.

Early recognition and management of these patients may avoid some of the chronic long-term morbidity often associated with this condition; however, late relapse because of noncompliance is a substantial risk and children should be followed up long term.

SRUS is thought to be part of the bigger disease process known as mucosal prolapse syndrome, which incorporates inflammatory cloacogenic polyps, inflammatory cap polyps, and gastric antral vascular ectasia. In fact, all these syndromes have the same histological features[61]. As a result of the wide endoscopic spectrum of SRUS and the fact that the condition may go unrecognized or, more commonly, misdiagnosed, it is crucial to take biopsy specimens from the involved area to make a positive confirmation of the diagnosis and to exclude other diagnoses including malignancy[31,62].

The exact etiology is unknown[30,63,64] but it has also been noted that this syndrome is often associated with trauma resulting in focal ischemia and ulceration, pelvic floor disorders[64], mucosal prolapse[14,52,64,65] and/or a larger systemic process[66]. Also, it has been associated with perineal descent, nonrelaxing puborectalis syndrome, and rectal prolapse[21,52]. In children, paradoxical contraction of pelvic floor and external anal sphincter muscles contributes to constipation, rectal prolapse, ischemia and finally rectal ulcer.

Diversity of the clinical presentation of SRUS requires a high index of suspicion of both the clinician and the pathologist for the definite diagnosis[30].

The clinicopathological similarities between SRUS and inflammatory bowel disease and the limited pediatric experience of these conditions may lead to difficulties in differentiating these conditions, and could result in under-reporting of SRUS in this age group.

It can present as more common childhood intestinal conditions such as inflammatory bowel disease or constipation, causing difficult-to-manage lower gastrointestinal symptoms. Also, it may present as polypoid mass lesions[67]. A biopsy is required for confirmation of diagnosis, because ulceration may not be apparent at the time of endoscopy. SRUS should be considered in children presenting with rectal bleeding, mucorrhea and excessive straining during defecation.

Biofeedback[44,45,47,48], sucralfate enema[15,50] and surgery seem to be ideal strategies because they aim to correct the underlying processes[57,58]. Behavioral modification or biofeedback therapy improves both rectal blood flow and symptoms and includes bowel habit training, avoiding excessive straining, and normalization of pelvic floor coordination[46-48,68,69]. Surgery is indicated in children with persistent bleeding per rectum not amenable to medical therapy and includes rectopexy, excision of ulcer, and rarely colostomy[42-44,56]. In children, a multitude of procedures have been advocated for rectopexy and a cure rate of at least 90% has been reported for posterosagittal rectopexy[43]. Also, El-Hemaly *et al*[70] have reported that the results of surgery and biofeedback are satisfactory in comparison to conservative treatment.

Most patients with SRUS in childhood have a satisfactory outcome using a simple behavioral modification approach. Ongoing follow-up to reinforce behavioral modification is important and may avoid long-term, treatment-resistant disease into adulthood. Despite the previous reports about SRUS in children that indicate low prevalence of the disease in childhood, recently we have been faced with higher prevalence rates in this age group. It seems that detailed and effective diagnosis methods such as endoscopy and histological examinations, as well as more attention by clinicians to this syndrome in children, have improved the diagnosis rate of the disease. Despite this being a benign condition in children, morbidity remains a problem as reflected by persistence of symptoms especially bleeding per rectum. Therefore, we are faced with an important condition that needs more attention and attempts for prevention and treatment.

More studies are needed to evaluate all of the aspects of the syndrome in children and to recommend the best treatment protocol. Every child with SRUS must be assessed individually using all modalities of investigation to define clearly the underlying pathophysiology, and to select the appropriate treatment strategies.

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S- Editor Gou SX **L- Editor** Kerr C **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v18.i45.6546

World J Gastroenterol 2012 December 7; 18(45): 6546-6551
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

Yu-Yuan Li, Professor, *Series Editor*

Genetic and epigenetic variants influencing the development of nonalcoholic fatty liver disease

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Author contributions: Li YY solely contributed to this paper.
Supported by The Grants from Guangzhou Municipal Bureau of Health, China, No. 2008-Zdi-01 and 2009-ZDi-03
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Received: April 19, 2012 Revised: July 24, 2012
Accepted: August 14, 2012
Published online: December 7, 2012

Abstract

Nonalcoholic fatty liver disease (NAFLD) is common worldwide. The importance of genetic and epigenetic changes in etiology and pathogenesis of NAFLD has been increasingly recognized. However, the exact mechanism is largely unknown. A large number of single nucleotide polymorphisms (SNPs) related to NAFLD has been documented by candidate gene studies (CGSs). Among these genes, peroxisome proliferatoractivated receptor-γ, adiponectin, leptin and tumor necrosis factor-α were frequently reported. Since the introduction of genome-wide association studies (GWASs), there have been significant advances in our understanding of genomic variations of NAFLD. Patatin-like phospholipase domain containing family member A3 (PNPLA3, SNP rs738409, encoding I148M), also termed adiponutrin, has caught most attention. The evidence that PNPLA3 is associated with increased hepatic fat levels and hepatic inflammation has been validated by a series of studies. Epigenetic modification refers to phenotypic changes caused by an adaptive mechanism unrelated to alteration of primary DNA sequences. Epigenetic regulation mainly includes microRNAs (miRs), DNA methylation, histone modifications and ubiquitination, among which miRs are studied most extensively. miRs are small natural single stranded RNA molecules regulating mRNA degradation or translation inhibition, subsequently altering protein expression of target genes. The miR-122, a highly abundant miR accounting for nearly 70% of all miRs in the liver, is significantly under-expressed in NAFLD subjects. Inhibition of miR-122 with an antisense oligonucleotide results in decreased mRNA expression of lipogenic genes and improvement of liver steatosis. The investigation into epigenetic involvement in NAFLD pathogenesis is just at the beginning and needs to be refined. This review summarizes the roles of genetics and epigenetics in the development of NAFLD. The progress made in this field may provide novel diagnostic biomarkers and therapeutic targets for NAFLD management.

Key words: Nonalcoholic fatty liver disease; Epigenetic; MicroRNA; Methylation

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Li YY. Genetic and epigenetic variants influencing the development of nonalcoholic fatty liver disease. *World J Gastroenterol* 2012; 18(45): 6546-6551 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i45/6546.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i45.6546

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is one of the most common forms of chronic liver diseases and a cause of elevated serum aminotransferases worldwide. The prevalence of NAFLD in the general population of Western countries ranges from 20% to 30%[1-3]. Due to the alterations of diet structure and life style, the prevalence of NAFLD in developing countries has been increasing rapidly[4]. Recent studies, including one from our group indicate that the prevalence of NAFLD in Chinese population is about 15%[5-7]. The term NAFLD encompasses a morphological spectrum of diseases, ranging from simple fatty liver (SFL) to nonalcoholic steatohepatitis (NASH) and hepatic cirrhosis, which may progress to hepatocellular carcinoma (HCC). SFL generally has a benign prognosis. Only a minority of them develop NASH, which is characterized by inflammation, fibrosis and liver cell injury[8,9].

NAFLD has been shown to be associated with metabolic syndrome (MetS), which comprises obesity, type 2 diabetes, dyslipidemia and high blood pressure with insulin resistance being the central mechanism. NAFLD is presently considered the hepatic manifestation of MetS[5,6,8,10].

It is generally believed that environmental and genetic factors interact to produce NAFLD phenotype and determine its progression. However, the detailed pathogenesis that determines which individual develops NAFLD remains unclear. Recently, the emerging field of epigenetics shed lights on the pathogenesis of chronic liver disease including NAFLD[11,12]. Elucidation of genetic and epigenetic factors that predispose an individual to NAFLD may lead to development of noninvasive biomarkers for early diagnosis of NAFLD and may allow early preventive and therapeutic strategies for the people at the high risk. This review summarizes recent contributions to the field of the genetic and epigenetic variations that influence the development of NAFLD.

GENETIC VARIATIONS

Candidate gene studies

The genetic variations may result in conformational changes in the protein structures and functions of the genes. NAFLD is an exceedingly complex genetic disorder. Before 2008, the candidate genes based on the prior knowledge of MetS and NAFLD pathophysiology were selected for investigation[11,13]. In comparison with NAFLD, the relationships between the genotypes and phenotypes of MetS have been examined more extensively. A large number of single nucleotide polymorphisms (SNPs) at the genes encoding proteins involved in insulin resistance has been revealed to be associated with the development of MetS[14,15]. As there is substantial overlap in the pathogenesis of NAFLD and MetS, theoretically, many variations in candidate genes related to MetS may contribute to the pathogenesis of NAFLD: first, genes related to insulin resistance, such as adiponectin, resistin, insulin receptor, and peroxisome proliferatoractivated receptors-γ (PPAR-γ); second, genes influencing hepatic free fatty acid metabolism, such as hepatic lipase, leptin (or leptin receptor), adiponectin, microsomal triglyceride transfer protein, phosphatidylethanolamine N-methyltransferase (PEMT), PPAR-γ, cytochrome P 450, 2E1 and 4A; third, cytokine-related genes, such as tumor necrosis factor-α (TNF-α) and interleukin-10; fourth, genes affecting liver fibrogenic pathways, such as leptin, adiponectin, transforming growth factor beta1, connective tissue growth factor and angiotensinogen; and finally, genes encoding endotoxin receptors and oxidative stress responses, such as CD14, superoxide dismutase-2 and toll-like receptor-4. Among these genes, PPAR-γ, adiponectin, leptin and TNF-α were frequently reported in the field of MetS as well as NAFLD[11,13,16]. It is noted that one gene may have a number of SNPs at several nucleotide loci. For example, the SNPs at the *PPAR-γ* gene involved in MetS may occur at the loci of C-681G, C-689T, Pro12Ala, G67222A, A69208G, G81556T, T95872C, T115432G, C127599T and C161T, but only a few of them have been investigated extensively[17,18].

There is evidence supporting the theory that these genetic factors account for considerable variability in susceptibility to NAFLD. The SNPs may increase or decrease the function of the target genes and their encoding proteins. We have previously demonstrated that many candidate genes' SNPs mentioned above are associated with susceptibility to NAFLD. Some showed positive relationships (increased risk), i.e., TNF-α-238, adiponectin-45, leptin-2548, PPAR-γ-161 and PEMT-175. Other SNPs demonstrated a negative association (decreased risk), i.e., adiponectin-276 and hepatic lipase-514. Two were not relevant, i.e., TNF-α-380 and PPAR-γ coactivator-1a-482[19]. Gene variations might affect the pathogenesis of NAFLD *via* blood cytokines (such as leptin and adiponectin) and insulin resistance pathways[19,20]. Although many pathobiological candidacies of SNPs were reported, most studies in literature have not been well validated by larger replication cohorts. The findings in candidate gene studies might be influenced by specific ethnic groups or environmental conditions.

Genome-wide association studies

Since the introduction of genome-wide association studies (GWASs) to investigate genomic variations, there have been significant advances in our understanding of human genome and its clinical sequelae over a range of diseases. More than 3.1 million SNPs have been identified so far. The International HapMap Project (http://hapmap.ncbi.nlm.nih.gov) has characterized patterns of SNPs across individuals from diverse ethnic backgrounds[16,21,22]. Although a number of GWASs has been published in the field of MetS (type 2 diabetes and insulin resistance)[23], and other liver diseases (HCC, hepatitis B, hepatitis C, drug-induced liver injury and primary biliary cirrhosis)[16,24,25], only a few studies were carried out on NAFLD.

In 2008, the first GWAS on NAFLD was reported by Romeo *et al*[26]. In this population-based study, noninvasive

proton magnetic resonance spectroscopy (1H-MRS) was applied to assess hepatic steatosis. Totally, 2111 individuals comprising a mixed population of Hispanic, African American, and European American were enrolled. Non-synonymous sequence variations of 9229 SNPs were identified in NAFLD group compared with normal controls. An allele of patatin like phospholipase domain containing family member A3 (PNPLA3, SNP rs738409, encoding I148M), also termed adiponutrin, on chromosome 22 was shown to be strongly associated with increased hepatic fat levels and hepatic inflammation. This allele was most common in Hispanics, the group most susceptible to NAFLD, with hepatic fat content being more than twofold higher in G homozygous subjects than in non-carriers. G allele frequency was lower in people of European descents and lowest in African Americans, the group found to have the lowest level of hepatic triglyceride accumulation. These findings were validated by another GWAS. Totally 1117 individuals with histologically confirmed NAFLD were genotyped for six SNPs relevant to hepatic fat levels and liver enzymes. PNPLA3 was significantly associated with steatosis, portal inflammation, lobular inflammation, Mallory-Denk bodies, NAFLD activity score and fibrosis[27]. Subsequently, the extension of the hepatic phenotype associated with the PNPLA3 genotype was independently replicated in both adult and pediatric subjects with simple steatosis, NASH and NASH-related fibrosis using different laboratory techniques[28-33]. There was evidence that carriers of PNPLA3 exhibited more severe steatohepatitis and higher levels of fibrosis. PNPLA3 was consistent with the concept of NASH rather than the broader features of the MetS, such as body mass index, dyslipidemia, and type 2 diabetes mellitus. The influence of PNPLA3 on hepatic steatosis was not through insulin resistance pathway as assessed by hyperinsulinaemic, euglycaemic clamp and oral glucose tolerance testing[27-29,32,34,35]. Recently a meta-analysis enrolling 16 studies (2937 subjects) was performed to evaluate the association of PNPLA3 with NAFLD. The results showed that PNPLA3 exerted a strong influence not only on liver fat accumulation (the GG homozygous subjects had a 73% higher lipid fat content compared with CC ones), but also on higher susceptibility to liver disorders (GG homozygous subjects had 3.24-fold higher risk of higher necro-inflammatory scores and 3.2-fold higher risk of developing fibrosis compared with CC homozygous ones). The PNPLA3 GG genotype *vs* the CC genotype was associated with a 28% increase in alanine transaminase (ALT) level. NASH was more frequently observed in GG than in CC homozygous subjects (odds ratio 3.488, 95%CI: 1.859-6.545). Nevertheless, carrying GG alleles did not seem to increase the risk of severe histological features[36]. In a clinical study recruiting 302 subjects with 1H-MRS-confirmed NAFLD whose genotyping was determined with TaqMan polymerase chain reaction (PCR), a SNP (rs767870) at adiponectin receptors 2 (ADIPOR2), but not at *ADIPOR1* and *PPAR* gene, was found to link to a higher liver fat content. In this study, PNPLA3 was not tested[37].

Although most studies supported the association between PNPLA3 and NAFLD, a few reports failed to validate this finding. In a GWAS enrolling 236 women with biopsy-confirmed NAFLD, no association for any feature of NAFLD with PNPLA3 was found. Another SNP (rs2645424) on chromosome 8 in the farnesyl diphosphate farnesyl transferase 1 gene, generating an enzyme with a role in cholesterol biosynthesis, was identified to relate to the severity of NAFLD histology including NAFLD activity score, liver fibrosis, lobular inflammation as well as increased ALT[38].

The results from GWASs shed light on the understanding of the genetics in NAFLD, as the loci identified are frequently novel and have not previously been implicated. However, such findings require further detailed studies both to determine the activity and to validate the causality, as neither biological functions nor pathogenic mechanisms of these genetic variations are known.

EPIGENETIC MODIFICATIONS

During the past decade, the role of epigenetic mechanisms in the pathogenesis of disease has been increasingly recognized. Epigenetic modification, mainly including microRNAs (miRNAs, miRs), DNA methylation, histone modification and ubiquitination, refers to phenotypic changes caused by the mechanism that is unrelated to changes in the underlying DNA sequence. As an adaptive mechanism to alteration of genetic and environmental signal patterns and epigenetic regulation, which allows fine-tuning gene expression, is essential for the proper maintenance of cellular homeostasis. Disruption of the balance will lead to the development of a wide range of disorders. So far, epigenetic research has mainly focused on cancer, cardiovascular disease, mental illness and autoimmune disease. The roles of epigenetics in the pathogenesis of NAFLD are largely unknown[39]. Among epigenetic modifications, miRs are studied most extensively in NAFLD. miRs are small naturally occurring single stranded RNA molecules regulating mRNA degradation or translation inhibition, subsequently altering protein expression of target genes. One miR can target multiple genes (multiplicity) and multiple miRs may target a single gene (cooperativity). Since the first discovery in 1993, many miRs in various organisms have been determined. To date, more than 1420 miRs have been identified in humans (miRBase v17). (http://www.mirbase.org/)[40,41]. The expression of miRs is both organ-specific and dependent on the stage of development. miRs influence at least one-third of all human transcripts and are known regulators of important cellular processes, e.g., cell metabolism, cell proliferation, apoptosis, immune function, tissue development and differentiation[42,43]. It has recently been shown that some 100 miRs are differentially expressed in human NASH. These miRs have diverse functions involved in the pathogenesis of NAFLD, including metabolisms of lipid and glucose, regulations of the unfolded protein response, endoplasmic reticulum stress, oxidative stress, cellular differentiation, inflammation, apoptosis and so on[44,45]. In a

clinical study, the miR profiles of 15 patients with biopsy-proven NASH and 15 controls with normal liver histology were investigated. Out of a total of 474 tested miRs, 46 were differentially expressed in NASH with 23 being up-regulated (in particular, miR-34a and miR-146b), and 23 being down-regulated (in particular, miR-122). These differentially expressed miRs were further validated by quantitative real-time PCR[45].

The miR-122, a highly abundant miR in the liver, has caught most attention in liver diseases. Accounting for nearly 70% of all miRs in the liver, miR-122 is significantly under-expressed (63%) in NASH subjects compared to controls[45,46]. In addition to its role in lipid and cholesterol metabolism, miR-122 has been shown to promote adipocyte differentiation[42]. Subsequently, the roles of miR-122 in the pathogenesis of NAFLD were confirmed by a number of studies. Inhibition of miR-122 in a diet-induced obesity mouse model with an antisense oligonucleotide treatment resulted in decreased mRNA expression of acetyl-coenzyme-A carboxylase-2, fatty acid synthetase, sterol regulatory element binding proteins 1-c, 2, stearoyl-CoA desaturase and 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG CoA) reductase, all of which were key lipogenic factors in human NASH. The histology also showed substantial improvement in liver steatosis[47]. The results were validated by another study in mice, in which the plasma cholesterol level, hepatic fatty-acid and cholesterol synthesis rate as well as HMG-CoA reductase level were significantly decreased after silencing miR-122[42]. All these findings strongly suggested the significance of miR-122 in the regulation of lipid metabolism and the contribution to the development of NAFLD. A further study suggested that miR-122 was closely linked to the output system of the circadian clock by regulating circadianly expressed genes[48]. Besides miR-122, some miRs have been demonstrated to be involved in NAFLD development. miR-34a and miR-146b were shown to be significantly over-expressed (99% and 80%, respectively) in human NASH[45]. The expression of miR-335 in the liver and white adipose tissue was up-regulated in mice. The increased miR-335 expression was associated with increased body, liver and white adipose tissue weight, as well as elevated hepatic triglyceride and cholesterol levels. Furthermore, hepatic miR-335 level was closely correlated with the expression of adipocyte differentiation markers, i.e., PPAR-α and FAS in adipocyte[49]. The presence of miR-181d significantly decreased lipid droplets in the liver (60%), and subsequently reduced cellular triglyceride and cholesterol[50]. miR-10b regulated steatosis level through PPAR-α pathway in a steatotic hepatocyte (L02 cell line) model. The post-transcriptional regulation of PPAR-α by miR-10b was maintained by a single binding site[51].

Aberrant methylation patterns of genomic DNA have been studied in many diseases. Hypermethylation of CpG islands is generally associated with gene silencing, and hypomethylation of global genomic DNA affects genomic stability. Hypermethylation of multiple genes in CpG islands has been demonstrated in human HCC, in which CpG island methylator phenotype was involved in the promoter hypermethylation of multiple genes[52]. However, the relation of DNA methylation to NAFLD development has not been well documented. A recent study enrolling 63 NAFLD patients confirmed by liver biopsies and 11 controls showed a tight interaction between the presence of NAFLD and hepatic DNA methylation of CpG in PPAR-γ coactivator 1α (PPARGC1A) and mitochondrial transcription factor A (TFAM) promoters. The proportion of DNA methylation in PPARGC1A and TFAM was significantly higher in the NAFLD livers than in the controls. However, the histological severity and activity scores of NAFLD were not correlated to methylation level and methylated DNA/unmethylated DNA ratio either in PPARGC1A or TFAM promoter[53]. The development of hepatic steatosis in a mouse model was accompanied by prominent epigenetic abnormalities, which comprised pronounced loss of genomic and repetitive sequences cytosine methylation, increased level of repeat-associated transcripts, aberrant histone modifications and alterations in expression of the maintenance DNA methyltransferase 1 (DNMT1) and *de novo* DNMT3A proteins in the livers[54].

Ubiquitination and sumoylation (sumo: abbreviation of small ubiquitin-like protein) are recently demonstrated to be novel forms of post-translational modifications (PTMs). PTMs of transcription factors through the course of protein processing play important roles in controlling many biological events[55]. The research of ubiquitination related to NAFLD is just at the beginning. In a study investigating the hepatic gene networks in morbidly obese patients with NAFLD, hepatic fibrosis signaling was found to be the most significant pathway in the up-regulated NAFLD gene cluster, whereas the endoplasmic reticulum stress and protein ubiquitination pathways to be the most significant pathways in the down-regulated *NAFLD* gene cluster[56]. Besides ubiquitination, transcription factors can undergo several types of PTMs, including acetylation, phosphorylation, and glycosylation. Little is known about their role in NAFLD so far[55].

In conclusion, environmental and genetic factors interact to produce NAFLD phenotype and to determine its progression. This review summarizes the current knowledge of genetic and epigenetic determinations on NAFLD. Genetic variations (e.g., SNPs) account for only a small fraction of environmental and heritable disease risks, whereas epigenetic modifications (e.g., miRs, DNA methylation histone modifications and ubiquitination) affect a bigger proportion of disease phenotypes. The investigation into the potential roles of epigenetics in NAFLD is just at the beginning and needs to be refined. The accumulation of genetic and epigenetic knowledge related to NAFLD has provided novel insight into disease pathogenesis, and may help to develop new diagnostic biomarkers and therapeutic targets for NAFLD management.

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S- Editor Shi ZF **L- Editor** Ma JY **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v18.i45.6552

World J Gastroenterol 2012 December 7; 18(45): 6552-6559
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Way back for fructose and liver metabolism: Bench side to molecular insights

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Supported by Fundació Privada Catalana de Nutrició i Lípids; and Grant SAF2010-15664 from the Spanish Ministry of Science and Innovation
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Received: June 20, 2012 Revised: September 26, 2012
Accepted: September 29, 2012
Published online: December 7, 2012

Abstract

The World Health Organization recommends that the daily intake of added sugars should make up no more than 10% of total energy. The consumption of sugar-sweetened beverages is the main source of added sugars. Fructose, together with glucose, as a component of high fructose corn syrups or as a component of the sucrose molecule, is one of the main sweeteners present in this kind of beverages. Data from prospective and intervention studies clearly point to high fructose consumption, mainly in the form of sweetened beverages, as a risk factor for several metabolic diseases in humans. The incidence of hypertension, nonalcoholic fatty liver disease (NAFLD), dyslipidemia (mainly hypertriglyceridemia), insulin resistance, type 2 diabetes mellitus, obesity, and the cluster of many of these pathologies in the form of metabolic syndrome is higher in human population segments that show high intake of fructose. Adolescent and young adults from low-income families are especially at risk. We recently reviewed evidence from experimental animals and human data that confirms the deleterious effect of fructose on lipid and glucose metabolism. In this present review we update the information generated in the past 2 years about high consumption of fructose-enriched beverages and the occurrence of metabolic disturbances, especially NAFLD, type 2 diabetes mellitus, and metabolic syndrome. We have explored recent data from observational and experimental human studies, as well as experimental data from animal and cell models. Finally, using information generated in our laboratory and others, we provide a view of the molecular mechanisms that may be specifically involved in the development of liver lipid and glucose metabolic alterations after fructose consumption in liquid form.

Key words: Obesity; Metabolic syndrome; Hypertension; Dyslipidemia; Nonalcoholic fatty liver disease; Clinical studies; Experimental studies; Sweetened beverages

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Rebollo A, Roglans N, Alegret M, Laguna JC. Way back for fructose and liver metabolism: Bench side to molecular insights. *World J Gastroenterol* 2012; 18(45): 6552-6559 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i45/6552.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i45.6552

INTRODUCTION

At the end of 2011, the United Nations declared that, for the first time in the history of humanity, non-communicable diseases had outpaced infectious diseases as the main threat to human health globally. Among them, cardiovas-

cular diseases associated with metabolic disorders, such as obesity, metabolic syndrome, and type 2 diabetes mellitus, are of paramount importance. Changes in human dietary habits in recent decades have led to the consumption of hypercaloric diets that are rich in saturated fats and simple sugars (sucrose, glucose and fructose). This, combined with decreased physical activity, is one of the key factors contributing to the ever-increasing prevalence of metabolic disorders. This situation recently prompted Lustig *et al*[1] to request the legal regulation of foodstuffs containing added sugars in a way similar to the control of tobacco and alcohol.

The World Health Organization recommends that the daily intake of added sugars should make up no more than 10% of total energy[2]. The consumption of sugar-sweetened beverages is the main source of added sugars[3]. Fructose, together with glucose, as a component of high fructose corn syrups (HFCSs) or as a component of the sucrose molecule, is mainly responsible for the metabolic disturbances associated with excessive consumption of added sugars. We recently reviewed evidence from experimental animals and human data that confirms the deleterious effect of fructose on lipid and glucose metabolism[4]. Given the relevance of this issue to public health policies, in this review we update information on the effects of fructose on human health. We focus also on new experimental data from our laboratory and others on molecular mechanisms involved in the disturbance of liver metabolism by fructose.

FRUCTOSE: THE BENCH SIDE

Data from prospective and intervention studies clearly point to high fructose consumption, mainly in the form of sweetened beverages, as a risk factor for several metabolic diseases in humans. The incidence of hypertension, nonalcoholic fatty liver disease (NAFLD), dyslipidemia (mainly hypertriglyceridemia), insulin resistance, type 2 diabetes mellitus, obesity, and the cluster of many of these pathologies in the form of metabolic syndrome is higher in human population segments that show high intake of fructose. Adolescent and young adults from low-income families are especially at risk. We and others have recently reviewed the evidence of this relationship[4-8]. In the present review, we provide an overview of recent data, from 2011 onwards that has not been discussed previously (Table 1). For readers interested in recent reviews on this subject, particularly regarding fructose consumption, uric acid metabolism and hypertension, we refer to two excellent reviews published in 2011[9,10].

One of the ongoing controversies about fructose consumption in humans is related to the difficulty in identifying effects that are not strictly related to the simple consumption of an excess of daily calories. In a short (2 wk) dietary intervention study in NAFLD subjects, Browning *et al*[11] showed that carbohydrate restriction (< 20 g/d) was significantly more effective in reducing hepatic triglyceride content than the restriction of calories to 1200-1500 kcal/d (55% *vs* 28%, respectively), despite the fact that both interventions similarly reduced body weight (by about 4.3%). In a randomized intervention study comparing the consumption of sucrose-sweetened beverages (1 L/d for 6 mo) with other isocaloric beverages in obese subjects, Maersk *et al*[12] demonstrated that sucrose significantly increased triglyceride deposition, not only in liver, but also in skeletal muscle and visceral adipose tissue.

In another intervention study in healthy people who consumed a balanced diet supplemented with 150 g/d fructose or glucose, Silbernagel *et al*[13] showed that endogenous cholesterol synthesis was associated with visceral and liver fat content. However, in this study the strongest association was observed in glucose-consuming individuals. Nevertheless, in a well-conducted interventional study by Stanhope *et al*[14], subjects who consumed fructose (at 25% of energy requirements), either as such or as HFCS, but not glucose, showed an increased fasting concentration of low density lipoprotein (LDL) cholesterol. Fructose consumption also increased the 24-h triglyceride area under the curve and the fasting apolipoprotein (apo)B concentration.

In a prospective cohort study that analyzed 40 389 healthy men over 20 years of follow up, de Koning *et al*[15] clearly found an association between sugar-sweetened beverage consumption and an elevated risk of type 2 diabetes mellitus. Although it was suggested that fructose was mainly responsible for this association, Silbernagel *et al*[16] did not find any differences between fructose and glucose in the reduction of insulin sensitivity when these sugars were administered to 20 healthy subjects in a small intervention study. However, plasma triacylglycerol concentrations only increased significantly in the fructose group.

Fructose-induced obesity is closely related to type 2 diabetes mellitus. In a well- conducted intervention study by Cox *et al*[17] in overweight/obese male and female subjects, consumption of fructose (at 25% of energy requirements for 10 wk), but not glucose, clearly led to significant decreases in net postprandial fat oxidation and resting energy expenditure, thus contributing to the build-up of excess energy substrates. Furthermore, in one of the population segments at high risk of fructose-related obesity, Maier *et al*[18] demonstrated that a significant reduction in fructose and/or general sugar intake over a short period of time (3 mo) in overweight and obese children may reduce the body mass index. Mainly through increases in visceral fat, fructose-induced obesity is positively associated in adolescents with cardiometabolic risk markers, such as systolic blood pressure, fasting glucose, homeostasis model assessment-estimated insulin resistance index, and C-reactive protein[19].

Cardiovascular accidents originate as thrombi deposits on atheromatous plaques, which obstruct blood circulation[20]. Atherosclerosis is promoted by dyslipidemia, hypertension, and chronic low-grade inflammation. Besides increasing plasma triglycerides and LDL cholesterol[14],

Table 1 Overview of fructose-related human studies

Authors	Subjects	Study characteristics	Sugar	Main results
Browning *et al*[11]	18 NAFLD (5 men, 13 women), BMI: 35 ± 7 kg/m^2	Intervention study 2 wk dietary carbohydrate and calorie restriction		Reductions in body weight (-4.6 ± 1.5 kg *vs* -4.0 ± 1.5 kg) and hepatic triglycerides (-55% ± 14% *vs* -28% ± 23%) were significantly greater with dietary carbohydrate restriction than with calorie restriction
Maersk *et al*[12]	60 overweight/obese nondiabetic subjects	Randomized intervention study Ingestion of 4 different drinks (1 L/d, SSB, isocaloric semiskim milk, aspartame-sweetened and water) for 6 mo	S	Daily intake of SSB with sucrose increased ectopic fat accumulation (liver, skeletal muscle) and lipids (blood cholesterol and triglycerides) compared with the other beverages
Silbernagel *et al*[13]	Healthy male (12) and female (8) adults	Dietary intervention study 150 g/d for 4 wk	F and G	Visceral and liver fat content associated to cholesterol synthesis Cholesterol synthesis appeared to be dependent on fructose/glucose intake
Stanhope *et al*[14]	48 adults, BMI 18-35 kg/m^2	Dietary intervention study Consumption of simple sugars at 25% of energy requirements for 2 wk	F and G	F consumption increased cardiovascular risk factors (AUC-Tg, fasting LDL and apo B) more than G
de Koning *et al*[15]	40 389 healthy men	Prospective cohort study 20 yr of follow-up of SSB and artificially sweetened beverages consumption	F, G and S SSB	After adjustment for several confounders, the hazard ratio for the association of SSB with incident type 2 diabetes was 1.24 for the comparison of the top with the bottom quartile of SSB intake
Silbernagel *et al*[16]	Healthy male (12) and female (8) adults	Dietary intervention study 150 g/d for 4 wk	F and G	Insulin sensitivity decreased in both intervention groups, while plasma triglycerides were increased in the F group
Cox *et al*[17]	Overweight/obese male (16) and female (15) adults	Intervention study 10 wk supplementation with SSB at 25% of energy requirements	F and G SSB	F-consuming subjects had a significant reduction in net postprandial fat oxidation and resting energy expenditure
Maier *et al*[18]	15 overweight/obese children (5-8 yr)	Dietary intervention study parental training to reduce dietary sugar content (–50% from baseline, 12 wk) and 12 wk of follow-up	F, G and S	Reductions in sugar intake were related to significant reductions in BMI and BMI standard deviation scores
Pollock *et al*[19]	559 adolescents (14-18 yr)	Association study of F intake and cardiometabolic risk factors	F	After adjustment, higher F consumption directly associated to BP, fasting glucose, HOMA-IR and C-reactive protein, and inversely to HDL-cholesterol and adiponectin. The introduction of visceral fat as a covariate attenuated these trends
Cox *et al*[21]	Overweight/obese male (16) and female (15) adults	Intervention study 10 wk supplementation with SSB at 25% of energy requirements	F and G SSB	Fasting concentrations of MCP-1, PAI-1 and E-selectin as well as postprandial concentrations of PAI-1 increased in subjects consuming F but not in those consuming G
Brown *et al*[22]	2696 people	Cross-sectional association study	F, G and S SSB	Direct and independent associations of SSB intake and BP Greater sugar-BP differences for persons with higher sodium excretion
Friberg *et al*[25]	61 226 women	Population-based cohort study 18.4 yr of follow-up of total sucrose, high-sugar-foods	F, G and S	Total sucrose intake and consumption of sweet buns and cookies was associated with increased risk of endometrial cancer
Ye *et al*[26]	737 non diabetic adults	Association study of sugar intake and cognitive function	F, G and S	Greater intakes of total sugars, added sugars and SSB beverages, but not of sugar sweetened solid foods, were significantly associated with lower MMSE scores, after adjusting for covariates

F: Fructose; G: Glucose; S: Sucrose; SSB: Sugar-sweetened beverages; BP: Blood pressure; NAFLD: Nonalcoholic fatty liver disease; BMI: Body mass index; MCP-1: Monocyte chemoattractant protein-1; PAI-1: Plasminogen activator inhibitor-1; HOMA-IR: Homeostasis model assessment-estimated insulin resistance; HDL: High-density lipoprotein; AUC-Tg: 24 h area under the curve for plasma triglycerides; LDL: Low-density lipoprotein; MMSE: Mini-mental state examination.

fructose seems to promote a proinflammatory milieu that favors atherosclerosis development. In an intervention study in overweight/obese subjects, Cox *et al*[21] demonstrated that fructose supplementation in liquid form (at 25% of energy requirements for 10 wk), but not glucose, clearly increases proinflammatory and prothrombotic mediators, such as monocyte chemoattractant protein-1, plasminogen activator inhibitor-1, and E-selectin. Furthermore, in a cross-sectional study including 2696 participants in the International Study of Macro/Micronutrients and Blood Pressure, Brown *et al*[22] found a direct association between sugar-sweetened beverage intake and systolic and diastolic blood pressure increases. Thus, fructose seems to contribute directly to increased prevalence in the three main risk factors for atherosclerosis-related cardiovascular diseases.

Besides the association between fructose consumption and common metabolic diseases, there is growing evidence of a relationship with other diseases, such as cancer and Alzheimer's disease, that are also closely connected to the cellular metabolic status[23,24]. Very recently, Friberg *et al*[25] analyzed data on total sucrose and high-sugar food consumption during 18.4 years of follow-up in 61 226 women. They found a direct association with increased risk of endometrial cancer. In addition, high sugar intake has recently been associated with lower cognitive function among middle-aged and older Puerto Ricans without diabetes, in an analysis of data from a substudy of the Boston Puerto Rican Health Study 2004-9[26]. Although a high fructose diet does not affect spatial water maze learning and memory in female rats[27], the presence of NAFLD, which is one of the main consequences of fructose consumption in men and experimental animals, seems to somehow impair hippocampal-dependent memory in male rats[28].

Thus, overall, it seems that a high intake of sugar-sweetened beverages containing fructose places a metabolic burden on humans that facilitates the development of metabolic and cardiovascular diseases. What molecular mechanisms are involved in the production of these effects by fructose?

FRUCTOSE: MOLECULAR INSIGHTS FROM ANIMAL STUDIES

Fructose administration, mainly in drinking water, to laboratory rats and mice reproduces almost all of the features of metabolic syndrome and associated diseases in humans. These include left ventricular hypertrophy[29,30], insulin resistance[30-33], hypertension and related hyperuricemia[34-36], NAFLD[37,38], and metabolic syndrome itself[39].

London *et al*[40] have investigated the role of increased 11-hydroxysteroid dehydrogenase type 1 in liver and visceral adipose tissue in rats after fructose, but not glucose, consumption. Their results indicate that deregulated local glucocorticoid production plays a role at the onset of fructose-induced obesity[40]. Morris *et al*[41] put forward the hypothesis that the timing of fructose intake, mainly during the daylight period, could induce a mismatch in caloric consumption that favors the development of obesity and other metabolic alterations, at least in C57BL mice. Furthermore, several possible hypotheses related to the development of NALFD by fructose consumption have been pursued, including increased oxidative and inflammatory stress through nitric oxide synthase induction[42] and tumor necrosis factor α production[43]. A very concise and interesting review on the issue of possible molecular mechanisms involved in fructose induced lipogenesis was published in 2011[44].

In the past few years, our laboratory has researched three main issues regarding the molecular effects of fructose on liver fat and glucose metabolism: (1) possible drug therapies for the prevention and/or correction of fructose-induced metabolic pathologies; (2) molecular mechanisms that are responsible for early induction of glucose intolerance in female rats, as a previous step to developing insulin resistance and type 2 diabetes mellitus; and (3) molecular mechanisms leading to reduced peroxisome proliferator-activated receptor (PPAR) expression and activity in livers of female rats.

NAFLD is by far the most common cause of liver dysfunction. It is a spectrum of diseases ranging from fatty liver (steatosis) to steatohepatitis[45]. To date, the only effective treatment for NAFLD is modest calorie restriction and gradual weight loss[46]. Statins, hypolipidemic drugs that act by inhibiting the hydroxymethyl-glutaryl-CoA reductase enzyme, can be safely used in NAFLD patients[47], and there is evidence of improved liver histology in NAFLD patients treated with atorvastatin[48,49]. In a recently published study, we proposed a possible molecular mechanism for the therapeutic effect of atorvastatin on NAFLD[50]. Besides its well-known anti-inflammatory effect[51,52], atorvastatin reduced the liver expression of fructokinase in male rats supplemented with a 10% w/v solution of fructose for 14 d. Fructose consumption induces the expression of liver fructokinase in experimental animals[53,54] and in NAFLD patients[55]. As fructokinase is essential in controlling fructose metabolism, its induction establishes a vicious circle that progressively increases the deleterious effect of fructose on liver metabolism. Atorvastatin effectively facilitates the breaking of this circle. It contributes to an increase in fatty acid metabolism[56] and to a reduction in fatty acid synthesis that is driven by increased carbohydrate response element binding protein (ChREBP) transcriptional activity[57,58], which are necessary to revert the deposition of triglycerides in liver tissue.

We used the same experimental model of rats supplemented with a 10% w/v solution of fructose for 14 d, to show that female rats were more sensitive to the deleterious effect of fructose on glucose homeostasis than male rats, as only females showed signs of glucose intolerance[54]. In the same study, we found a marked reduction in insulin receptor substrate (IRS)-2 in the livers of fructose-supplemented female rats. IRS-2 is the main transducer of insulin signaling in hepatic tissue[59]. We have further pursued research of molecular changes related to fructose consumption in liver. We have confirmed that female rats supplemented with liquid fructose for 14 d, but not 7 d, are glucose intolerant (as shown by glucose tolerance test; GTT). This situation correlates with a decrease in the amount of IRS-2 protein expressed in liver. The same animals showed a marked increase in mammalian target of rapamycin (mTOR) activity and mitogen-activated protein kinase (p38-MAPK) activity.

p38-MAPK is a stress-related kinase[60] whose activity can be increased by the metabolic burden imposed by fructose metabolism in hepatocytes through two mechanisms: increased activity of protein phosphatase A2[54,61]; and the presence of bacterial toxins in blood, as a result of fructose-related alteration of the intestinal barrier

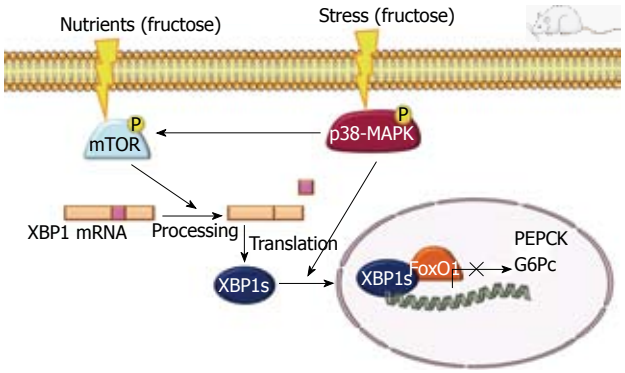

Figure 1 X-box-binding protein-1, an endoplasmic reticulum stress transcription factor, plays an essential role in maintaining plasma glucose concentration and glucose tolerance. Indeed, in the liver samples from the fructose-fed rats used in the study, there was a marked increase in the spliced form of X-box-binding protein (XBP)-1 mRNA and nuclear protein, in accordance with the increased activity of mammalian target of rapamycin (mTOR) activity and mitogen-activated protein kinase (p38-MAPK). Thus, although the decreased expression of insulin receptor substrate-2 in liver represents an impairment of insulin signaling, the increased expression and activity of XBP-1 could compensate for this deficit and maintain appropriate gluconeogenesis. PEPCK G6Pc: Phosphoenolpyruvate carboxykinase and glucose-6-phosphatase; FoxO1: Forkhead box protein O1.

permeability[43,62]. Furthermore, increased p38-MAPK activity, by phosphorylating the tuberous sclerosis 2 gene product or tuberin, could release its inhibitory activity on mTOR complex 1 (mTORC1)[63]. This would explain the observed increase in mTOR activity. The mTOR signaling pathway transduces information from different signals, such as growth factors, amino acids and energy overload of the cell[64]. Finally, as Guo *et al*[65] have shown that mTOR activation causes IRS-2 degradation, the increase in mTOR activity could be the final molecular factor resulting in a decreased liver expression of liver IRS-2 protein, as we have found[54].

Surprisingly, although female rats supplemented with liquid fructose for 14 d, had reduced liver expression of IRS-2, were hyperinsulinemic and showed an altered GTT, they were normoglycemic and their liver expression of gluconeogenic genes was unchanged (glucose-6-phosphatase) or even decreased (phosphoenolpyruvate carboxykinase). An explanation for this discrepancy can be found in a recent report indicating that X-box-binding protein (XBP)-1, an endoplasmic reticulum stress transcription factor, plays an essential role in maintaining plasma glucose concentration and glucose tolerance[66]. It has been described that mTORC1 activity increases the splicing of XBP-1[67], while p38-MAPK phosphorylates the spliced-derived protein, facilitating its nuclear localization and activity[68]. Indeed, in the liver samples from the fructose-fed rats used in our study, there was a marked increase in the spliced form of XBP-1 mRNA and nuclear protein, in accordance with the increased activity of mTOR and p38-MAPK. Thus, although the decreased expression of IRS-2 in liver represents an impairment of insulin signaling, the increased expression and activity of XBP-1 could compensate for this deficit and maintain appropriate gluconeogenesis (Figure 1). Data from skeletal muscle that indicate a deficit in adiponectin receptor and signaling in 14-d fructose-supplemented rats, could explain the fact that these animals do not have increased liver gluconeogenesis, but do have significant glucose tolerance impairment, as evaluated by an GTT.

We have previously shown that there is a state of leptin resistance in livers of male rats supplemented with liquid fructose. This results in increased binding of unphosphorylated active forkhead box protein (Fox)O1 to the transcription factor PPARα, which causes the inhibition of PPARα transcriptional activity and, as a consequence, reduces the liver capacity to oxidize fatty acids[57,58]. FoxO-1 is a transcription factor that is regulated by insulin and deeply involved in the control of liver gluconeogenesis[65]. Female rats equally supplemented with liquid fructose respond similarly with a reduction in liver PPARα activity and fatty acid oxidation. However, there is no involvement of leptin resistance and FoxO-1 interaction[54]. Thus, we have pursued the search for a possible molecular mechanism involved in the downregulation of the PPARα system in the liver of fructose-supplemented female rats.

ChREBP is a transcription factor responsible for inducing liver lipogenesis after carbohydrate ingestion[69]. We have previously reported that ChREBP is the main factor responsible for the increase in rat liver lipogenesis following fructose supplementation[50,54,57,58,70]. Unpublished results from our group indicate that there is also a close relationship between ChREBP activation and PPARα downregulation across different experimental settings (*in vivo* studies in female rats, cultured FaO and HepG2 hepatoma cells, primary cultures of human hepatocytes). It has been described that ChREBP controls the expression of regulator of G protein signaling (RGS) 16, a regulator of G protein signaling that inhibits hepatic fatty acid oxidation[71]. Although fructose markedly increased the mRNA level of RGS16 in livers of female rats, there was no change in the amount of the expressed protein. This suggests that increased expression of RGS16 is not involved in downregulation of the PPARα system. In rat hepatoma FaO cells cultured in the presence of a high concentration of fructose (25 mmol/L), we are performing knock-down experiments with siRNA against ChREBP to demonstrate clearly the direct involvement of ChREBP in the production of the fructose effect on the PPAR system. Confirmation of this hypothesis will indicate that fructose can simultaneously switch on liver fatty acid synthesis and switch off liver fatty acid catabolism by a single molecular mechanism: the intense activation of ChREBP. This would explain the effectiveness of fructose in inducing fatty liver and hypertriglyceridemia. We are also exploring possible mechanisms to explain why fructose stimulates the activity of ChREBP with such intensity. We have found that fructose supplementation markedly reduces the amount of the NAD-dependent deacetylase sirtuin 1 protein in livers of female rats,

but not males. This reduction increases the amount of acetylated ChREBP. As it has been shown that ChREBP hyperacetylation increases its transcriptional activity[72], the reduction of sirtuin 1 expression could be one mechanism involved in the intense activation of ChREBP by fructose in the liver of female rats.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v18.i45.6560

World J Gastroenterol 2012 December 7; 18(45): 6560-6570
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Role of gastrin-peptides in Barrett's and colorectal carcinogenesis

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Supported by Instituto de Salud Carlos Ⅲ with Grants FIS 08/1047 and CIBERehd; Instituto de Salud Carlos Ⅲ FI10/00167, to Chueca E
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Received: August 22, 2012 Revised: September 28, 2012
Accepted: October 16, 2012
Published online: December 7, 2012

Abstract

Gastrin is the main hormone responsible for the stimulation of gastric acid secretion; in addition, gastrin and its derivatives exert proliferative and antiapoptotic effects on several cell types. Gastrin synthesis and secretion are increased in certain situations, for example, when proton pump inhibitors are used. The impact of sustained hypergastrinemia is currently being investigated. *In vitro* experiments and animal models have shown that prolonged hypergastrinemia may be related with higher cancer rates; although, this relationship is less clear in human beings. Higher gastrin levels have been shown to cause hyperplasia of several cell types; yet, the risk for developing cancer seems to be the same in normo- and hypergastrinemic patients. Some tumors also produce their own gastrin, which can act in an autocrine manner promoting tumor growth. Certain cancers are extremely dependent on gastrin to proliferate. Initial research focused only on the effects of amidated gastrins, but there has been an interest in intermediates of gastrin in the last few decades. These intermediates aren't biologically inactive; in fact, they may exert greater effects on proliferation and apoptosis than the completely processed forms. In certain gastrin overproduction states, they are the most abundant gastrin peptides secreted. The purpose of this review is to examine the gastrin biosynthesis process and to summarize the results from different studies evaluating the production, levels, and effects of the main forms of gastrin in different overexpression states and their possible relationship with Barrett's and colorectal carcinogenesis.

Key words: Gastrin; Progastrin; Glycine-extended gastrins; C-terminal flanking peptide; Hypergastrinemia; Proton pump inhibitors; Colorectal cancer; Esophageal adenocarcinoma; Barrett's esophagus

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Chueca E, Lanas A, Piazuelo E. Role of gastrin-peptides in Barrett's and colorectal carcinogenesis. *World J Gastroenterol* 2012; 18(45): 6560-6570 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i45/6560.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i45.6560

INTRODUCTION

The polypeptide hormone gastrin was discovered in 1905 and described as a major stimulant of acid secre-

tion from the stomach antral mucosa. In the last few decades, several studies have reported on the role of gastrin in stimulating cell division and inhibiting apoptosis, suggesting that gastrin and its derivatives might promote carcinogenesis[1-5]. Gastrin and cholecystokinin (CCK) are members of a family of neuroendocrine peptides and are both physiological ligands of the CCK-B receptor (CCKBR).

Gastrin is secreted by antral G cells and interacts with the CCKBR on enterochromaffin-like (ECL) and parietal cells to induce gastric acid secretion.

Gastrin release from G cells is stimulated by the presence of food - mainly peptides[6] in the stomach, vagal release of gastrin releasing peptide, and an increase in stomach pH, as seen in achlorhydria[7]. *Helicobacter pylori* (*H. pylori*) infection is also known to cause hypergastrinemia, increasing mainly plasma levels of its amidated form gastrin-17. After eradication of the bacteria, plasma gastrin levels decrease to normal[8-10]. Gastrin release is inhibited by secretion of gastric acid, and this serves as a negative feedback control that prevents excess acid secretion. Low pH values in the stomach inhibit gastrin release by G cells, stimulating the secretion of somatostatin by antral D cells[11].

Gastrin is expressed in a variety of tissues under both normal and pathological conditions. Its main site of production are G cells from the antral mucosa, but it is also synthesized at lower levels in duodenal mucosa, fetal and neonatal pancreases, in pituitary corticotrophs, melanotrophs, and neurons, in spermatogenic cells, and in a variety of cancers.

The main products of the gastrin gene in the antrum are its amidated forms gastrin 17 and gastrin 34 (G17-NH_2 and G34-NH_2).

GASTRIN BIOSYNTHESIS

As with other peptide hormones, gastrin is synthesized initially as a large precursor molecule, which undergoes extensive post-translational modification prior to secretion. The gastrin gene spans 4.1 kb and is located on chromosome 17 (17q21). It produces a single mRNA (0.7 kb), which encodes the 101 amino acid precursor, preprogastrin[12]. Preprogastrin is translated at the endoplasmic reticulum, where the signal peptide is removed by signal peptidase, giving rise to progastrin (80 amino acids)[13]. Progastrin (PG) then progresses through the Golgi complex.

If the cell has a regulated secretory pathway, as with differentiated endocrine cells such as G-cells in the antrum, progastrin is fully processed and transported by secretory granules. It is then released by exocytosis, which is induced by secretagogues after G-cell stimulation. This is the secretory pathway of most of the amidated products, because the enzymes and conditions necessary for the processing of the immature gastrin forms are found inside secretory granules from the Golgi stack.

Progastrin is cleaved at paired amino acids by endoproteases belonging to the prohormone convertases (PC) family. PC1/3 cleavages at the dibasic sites arginine36-arginine37 and arginine73-arginine74 lead to the formation of an intermediate, which undergoes processing by carboxypeptidase E and yields glycine-extended gastrins (G-Gly) and the C-terminal flanking peptide (CTFP). The peptidylglycine α-amidating monooxygenase converts G34-gly to its amidated form and PC2 cleaves at lysine53-lysine54, producing bioactive gastrins of varying sizes (e.g., gastrin-34 and gastrin-17)[13,14] (Figure 1) .

Preprogastrin derivates can also exit the cell *via* another pathway, known as the constitutive pathway. Molecules exiting cells *via* this pathway are transported in secretory vesicles that take their contents from the Golgi apparatus and continuously fuse with the plasma membrane. Intermediate products of gastrin processing are secreted mainly by this pathway since peptides exiting this pathway do not undergo extensive posttranslational processing.

Processing and final secretion of progastrin products differ markedly depending on the expression location. In healthy adults, the main gastrin production site is antroduodenal G-cells, so the proportion of circulating gastrins depends largely on the products exiting these cells. In G-cells, the regulated secretory pathway predominates; thus, these cells mostly secrete a mixture of amidated products (95%), including G17-NH_2 (85%-90%), G34-NH_2 (5%-10%), and a mix of gastrin-14, gastrin-52, gastrin-71, and short amidated C-terminal fragments[15]. The remaining 5% of the secreted products correspond to non-amidated processing intermediates (mainly progastrin and G-Gly).

Although the majority of gastrins secreted by G-cells correspond to the amidated G17 form, peripheral blood contains almost equal amounts of G17-NH_2 and G34-NH_2 because the metabolic clearance of large gastrins is slower than for smaller forms of the peptide[16-18].

On the other hand, the proportions of the gastrin intermediates may vary in certain gastrin overexpression states, such as when proton pump inhibitors (PPIs) are used or in the presence of gastrin-producing tumors. Most of these tumors are not able to completely process gastrin, resulting in less conversion to the mature peptide[19-22].

The causes of incomplete gastrin processing during hormone overexpression are still unclear; although, it has been proposed that it might be caused by saturation of the enzymes that catalyze progastrin modifications, leading to an inability to process increasing amounts of the gene product.

Another possible reason is the lack of a well-developed regulated pathway of secretion, as in some tumor cells. In that case, progastrin exits the cell *via* the constitutive pathway directly from the Golgi terminal.

GASTRIN RECEPTORS

The actions of amidated gastrins and CCK peptides are mediated by two different receptors: CCKA and CCKB

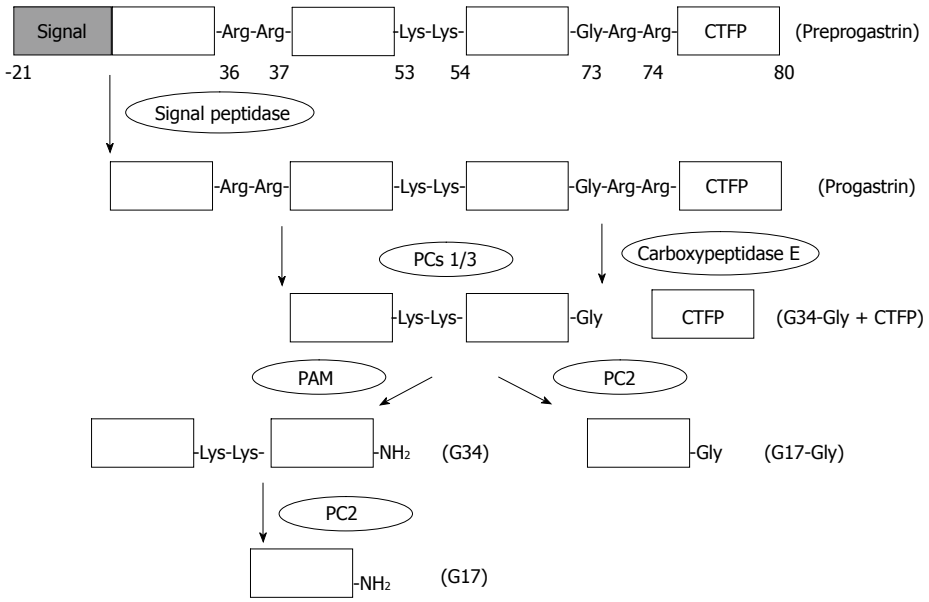

Figure 1 Main steps in preprogastrin processing in antral G cells. Arg: Arginine; Lys: Lysine; CTFP: C-terminal flanking peptide; PC: Prohormone convertases; PAM: Peptidyl-glycine α-amidating monooxygenase.

receptors, which differ pharmacologically by their affinity for gastrin (low for CCKA receptors and high for CCKB receptors)[23,24].

Gastrin and CCK peptides share a common C-terminal sequence, which has been well preserved during evolution. This conserved C-terminal active site is related to most of the known effects of these peptides, especially the tetrapeptide Trp-Met-Asp-Phe-NH_2. The specificity of the receptor binding and biological potency depends on N-terminal extensions of this common tetrapeptide.

Sulfation of the tyrosyl residue (in position six in gastrin peptides, counted from the C-terminal position, and in position seven in CCK peptides) determines the specificity for CCKA or CCKB receptors. The residue is totally sulphated in CCK peptides, so they are able to bind either CCKA or CCKB receptors with high affinity. It is partially sulphated in gastrin peptides, so they can only bind CCKB receptors.

Gastrin and CCK display similar affinities for the CCKB receptor; however, the gastrin concentration in plasma is 10- to 20-fold higher than CCK; therefore, CCKB receptors in the periphery are, in physiological terms, mainly receptors for gastrin.

The CCKB receptor has seven transmembrane domains and belongs to the superfamily of G-protein coupled receptors. CCKBR is abundantly expressed on enterochromaffin-like cells in the stomach, in the central nervous system and in some tumors, principally in the gastrointestinal tract.

Gastrin, at physiological levels, is the main mediator of meal-stimulated acid secretion. Once secreted by the antral G cells, gastrin is transported to the oxyntic mucosa of the stomach, where it interacts with the CCKBR on ECL cells, stimulating the release of histamine. Both gastrin and histamine then interact with the parietal cells, through the gastrin CCKB and histamine H_2 receptors to induce gastric acid secretion[25].

Only amidated gastrins exert their effects through CCKBR activation, while intermediate precursors such as progastrin or G-Gly interact with other receptors[3,26-28].

Most PG effects are mediated *via* the monomeric 36 kDa form of the annexin Ⅱ receptor (ANXⅡ)[29,30]. ANXⅡ is a multi-functional protein that binds acid phospholipids and actin with similar affinity. It's expressed abundantly in rejuvenating cells, but not in quiescent cells; in addition, its expression is increased in many human cancer cells, including colon and pancreatic, and it's expressed in normal intestinal epithelial cells[27,28]. ANXⅡ is absent in the brain and liver, which supports that it is only expressed in proliferating cells.

The majority of effects of G-Gly and CTFP appear to be mediated by a cellular receptor distinct from CCKBR[1,24,31-33]; yet, to date, the receptor or receptors remain unknown. Gly-G appears to be able to bind ANXⅡ, but it is still unclear whether its action is mediated *via* this interaction[1].

PPIS AND GASTRIN

PPIs are the most potent and widely used medications to reduce gastric acid secretion. These drugs are considered safe; although, some long-term side-effects have been identified, for example, all PPIs induce an increase in plasma gastrin levels. The reason for this increase remains unclear, but it may be due to the reduced activity in antral D-cells (shown by a three-fold decrease in antral somatostatin mRNA) in response to PPI-induced achlorhydria[7]. There is also an increase in plasma gastrin levels with other antacids such as H_2 receptor antagonists, but only after long-term use[34].

PPIs may induce a 2- to 4-fold increase in plasma gastrin[35,36] (mainly G17-NH_2 and G34-NH_2) with short-term treatment, whereas, in long-term therapy, some patients will develop marked hypergastrinemia (often exceeding 400 pmol/L). The antral mucosa levels of amidated gastrins and G-Gly are not affected by PPI treatment, but

Table 1 Studies assessing expression levels and/or biological effects of gastrin through its interaction with cholecystokinin-B receptor

Ref.	Specimen	CCKBR expression	G17 expression	G17 effects
Haigh *et al*[4]	Esophageal biopsies from healthy, esophagitis, BE and EAC patients; *Ex vivo* culture of BE cells; OE33(E)GR cells	CCKBR is expressed in 3/9 of healthy, 5/7 esophagitis, 10/10 BE and 7/12 EAC samples	Not assessed	G17 stimulates cell proliferation through CCKBR
Konturek *et al*[46]	Tumor and plasma samples from CRC patients; Plasma and normal colonic mucosa biopsies from healthy subjects	All the tumor samples showed CCKBR expression	CRC patients showed normal G17 plasma levels, and higher progastrin levels than healthy subjects; Celecoxib diminished plasma gastrin and progastrin levels	Not assessed
Smith *et al*[49]	Healthy colonic mucosa and colonic polyps biopsies	Normal colonic mucosa didn't show CCKBR expression; Most of the polyps analyzed showed CCKBR expression	Most of the polyps showed higher expression of the gastrin precursors than amidated forms	Not assessed
Harris *et al*[70]	Healthy esophagus and BE biopsies; OE19 and OE33 cell culture; OE21 cell culture	All three esophageal cancer cell lines express CCKBR; BE biopsies show higher CCKBR expression levels than normal esophageal biopsies	BE samples express higher gastrin levels than healthy esophageal biopsies	G17 increases activation of the antiapoptotic factor PKB/Akt, through CCKBR

BE: Barrett's esophagus; EAC: Esophageal adenocarcinoma; CCKBR: Cholecystokinin-B receptor; G17: Amidated gastrin-17; CRC: Colorectal carcinoma; OE19 and OE33 cells: Esophageal adenocarcinoma cell lines; OE21 cells: Esophageal squamous carcinoma cell line; OE33(E)GR cells: Esophageal adenocarcinoma cells permanently transfected with the human CCKB receptor.

a 6-fold increase in the antral progastrin concentration was observed after PPI therapy[37-39]. However, the gastrin levels in patients on PPIs are extremely variable, and not every subject will have a markedly increased plasma gastrin level after acid suppressive therapy.

PPIs rapidly stimulate antral gastrin secretion, but the overexpression of the gastrin gene, which is observed as increased gastrin mRNA concentrations in G-cells, is only seen after 24 h of achlorhydria[7].

To date, circulating levels of gastrin precursors have not been evaluated in response to PPI intake.

GASTRIN AND CARCINOGENESIS

As mentioned above, gastrin is a major stimulant of acid secretion in the stomach mucosa, but it also has effects in different tissues promoting cell division and inhibiting apoptosis. There is now growing evidence suggesting that elevated gastrin levels could favor the development of certain neoplasias, especially in the gastrointestinal tract[2,40-43]. To date, most of those studies have been focused on the possible relationship between elevated gastrin levels and colorectal and gastric cancers, but there is evidence that suggests a possible relationship with different tumors, even outside the gastrointestinal (GI) tract[19,20,22,44,45].

CCKBR has been observed in several tumor types, but expression of the receptor in human gastrointestinal cancers is controversial. Although some groups found CCKBR expression in many GI neoplasias[3,46], others found expression of the receptor in GI tumors only occasionally[23,29] (Table 1).

It is well established that some tumors produce their own gastrin and that gastrin can promote tumor growth in an autocrine manner[22,44,46-48], but there were conflicting findings from studies evaluating gastrin expression in tumors. This may be because initial attempts focused only on the amidated forms of gastrin. We now know that certain tumors, such as colorectal carcinoma (CRC), produce high levels of gastrin intermediates while the amidated forms are not affected[20,22]. Gastrin has been found in CRC extracts and also in adenomatous polyps[49], but not in healthy colonic mucosa. A similar pattern was found with esophageal adenocarcinoma and Barrett's esophagus (BE) (a premalignant condition that is a major risk factor for esophageal adenocarcinoma), where gastrin and its receptor were expressed at higher levels than in normal epithelium[4,50].

These observations suggest that activation of gastrin expression may be an early event in the adenoma-carcinoma or the metaplasia-carcinoma progression; thus, gastrin could favor neoplastic transformation.

Studies in animal models have demonstrated that a prolonged hypergastrinemic situation, such as in deep acid inhibition, is related to higher CRC rates and with gastric atrophy, metaplasia, gastric adenocarcinoma and carcinoid tumors[34,41,43,51] (Table 2). *In vitro* studies demonstrated that gastrin and its derivates increase the rate of cell proliferation and migration and reduce apoptosis, which are major steps in tumor development[1,3,28,44,52]. Although both *in vitro* and *in vivo* animal model studies seem to demonstrate an association between a rise in gastrin levels and a higher risk of cancer development this is still unclear in human beings. While some epidemiologic studies showed an association between, elevated gastrin levels after use of PPIs and stomach ECL and argyrophil cell hyperplasia, but couldn´t demonstrate that hypergastrinemia itself increases gastric adenocarcinoma rates[35,53-55], others found higher cancer rates (gastric and gastrointestinal overall) in hypergastrinemic patients[56,57] (Table 3).

Pernicious anemia could represent a human model

Table 2 Experimental studies in animal models exploring the impact of increased levels of gastrin peptides

Ref.	Animal model	Alteration on gastrin peptides levels	Hypergastrinemia effects
Cobb *et al*[2]	Fabp-wt mice; Fabp-mt mice	Fabp-wt mice express human PG in intestinal mucosa and Fabp-mt mice express a mutated form of human PG; Both mice show PG expression at similar levels as seen in hypergastrinemia	Mice overexpressing human PG (either the wild-type and the mutated form) are more likely to develop colonic tumors in response to AOM
Wang *et al*[5]	INS-GAS mice; hGAS mice	INS-GAS mice overexpress human amidated gastrin in the pancreatic islands; hGAS mice overexpress human PG in the liver	Both forms of gastrin showed similar proliferative effects on normal colonic mucosa
Havu *et al*[34]	Sprague-Dawley rats treated with ranitidine (2g/kg per day)	Rats showed a 3-fold increase in plasma gastrin levels	19/100 rats developed ECL carcinoids while no carcinoma was found in control animals
Watson *et al*[43]	$APC^{Min+/-}$ mice (model of multiple intestinal neoplasia) treated with omeprazole (75 mg/kg in a single oral dose)	Omeprazole increased only amidated gastrin plasma levels	PPI-induced hypergastrinemia reduced mice survival; Hypergastrinemia increased colonic adenomas proliferation; Hypergastrinemia did not increase the incidence of intestinal tumors
Ferrand *et al*[90]	MTI/G-Gly mice; hGAS mice	MTI/G-Gly mice overexpress human G-Gly throughout the gastrointestinal tract; hGAS mice overexpress human PG in the liver	Both G-Gly and PG strongly up-regulate Src, JAK2 and STAT3 activation; PG produced significantly great ERK and Akt pathways activation and TGF-α overexpression
Koh *et al*[95]	MTI/G-Gly mice	MTI/G-Gly mice overexpress human G-Gly throughout the gastrointestinal tract	Goblet cells hyperplasia and colonic hyperproliferation; Hypergastrinemia did not increase the incidence of GI tumors, but 3/10 mice developed bronchoalveolar carcinoma
Ottewell *et al*[98]	$G^{-/-}hg^{+/+}$ mice; $G^{-/-}hg^{-/-}$ mice	$G^{-/-}hg^{+/+}$ mice express human PG and no murine gastrin; $G^{-/-}hg^{-/-}$ mice do not express any forms of gastrin	PG increased colonic proliferation; PG exerts mitotic effects on colonic epithelia but does not seem to affect the small intestine epithelia

PG: Progastrin; AOM: Azoxymethane; ECL: Enterochromaffin-like cells; PPI: Proton pump inhibitors; G-Gly: Glycine-extended gastrins; JAK2: Janus-activated kinase 2; STAT3: Signal transducer and activator of transcription 3; ERK: Extracellular-signal regulated kinase; Akt: Protein kinase B; TGF-α: Transforming growth factor-alpha; GI: Gastrointestinal.

to assess effects of long-term hypergastrinemia, since it causes a long-term hypergastrinemia as a consequence of sustained achlorhydria[57]. Another human model of hypergastrinemia is Zollinger-Ellison syndrome. In this case, patients show higher rates of colonic proliferation[58], but not a higher risk for developing CRC[59].

Another study found a higher CRC incidence rate with higher serum gastrin levels[60]; while, one study found no association between PPI use and the risk of CRC[61].

It has been suggested that the discrepancy between results observed in human studies could be explained by the variability of hypergastrinemia after use of PPIs among patients[42], by differences in the duration of the follow-up period -since higher cancer rates have only been observed in long-time hypergastrinemic patients-, and by differences in the forms of gastrin being studied, given that most of the studies to date have been focused only on the amidated forms[22,62].

GASTRIN, BE AND ESOPHAGEAL ADENOCARCINOMA

Gastroesophageal reflux disease (GERD) is a chronic state in which part of the acidic stomach contents backs up into the esophagus and may cause inflammation of its epithelium. In most patients, this damaged epithelium is replaced by new squamous epithelium; however, in some subjects, this epithelium is substituted, through a metaplastic process, by an intestinal-type columnar epithelium. This condition is called BE, a premalignant state responsible for most esophageal adenocarcinoma cases (EAC). Patients with BE have a 30- to 40-fold higher risk for developing EAC than the general population[63].

In the last few decades, the incidence rates for this tumor have increased significantly[64], more than for any other type of cancer[65] in developed countries.

BE may represent a good model to study the involvement of hypergastrinemia in carcinogenesis, because frequently high levels of gastrin can be observed in BE patients. PPIs are the main pharmacological treatment for BE and the sequence of neoplastic transformation is well known.

In the pathological state caused by the damaging effects of acid contents from the stomach in the esophagus, it seems that an increase in gastric reflux pH would have a potential benefit for the patient. However, the benefits of these drugs in the management of GERD and BE are not clear. Normalization of intraesophageal pH clearly relieves gastroesophageal reflux symptoms[37], favoring differentiation and decreasing cell proliferation[66]; yet, there has been an increasing incidence of EAC in BE patients in the last few decades, despite generalized use of PPIs[67-69]. Studies addressing the potential

Table 3 Clinico-epidemiologic studies exploring the effects of proton pump inhibitors use in human beings

Ref.	Population studied	Treatment, dose and duration	Effects on gastrin levels	Physiopathological effects
Brunner *et al*[35]	143 patients with duodenal or stomach ulcer and GERD	Omeprazole 40 mg/d 1-5 yr	Plasma gastrin levels increased 4-fold after 4 mo of therapy	Hyperplasia of argyrophil cells from oxyntic mucosa; No increase in dysplasia or neoplasia rates was observed
Klinkenberg-Knol *et al*[37]	91 GERD patients	Omeprazole 20-40 mg/d 5 yr	Median serum gastrin levels increased from 60 to 162 ng/L and reached a plateau during maintenance treatment	Esophagitis symptoms ameliorated; Gastric hyperplasia rates increased from 2.5% at the beginning of the study to 20% at last biopsy
Nemeth *et al*[39]	10 patients with oesophagitis	Omeprazole 20 mg/d 6-8 wk	Plasma levels of amidated gastrins increased from 18 to 48 pmol/L; Antral levels of progastrin increased 6-fold while amidated gastrins and G-Gly remain unaltered	Not assessed
Wang *et al*[42]	82 BE patients; 13 GERD patients	All patients were on PPI therapy, once or twice daily during a median time of 74 mo	The median serum gastrin levels (40 pmol/L) was not related to the degree of dysplasia in BE	Higher serum gastrin levels were associated with high grade dysplasia and adenocarcinoma
Creutzfeldt *et al*[53]	74 patients with esophagitis or peptic ulcer	Omeprazole 40 mg/d 1-5 yr	Plasma gastrin levels increased 4-fold in 23% of patients	Patients with higher serum gastrin levels developed hyperplasia of the gastric argyrophil cells; This hyperplasia may not necessary be related to high gastrin levels
Kuipers *et al*[54]	177 GERD patients	105 patients treated with omeprazole 20-40 mg/d 5 yr; 72 patients treated with fundoplication	Not assessed	Patients treated with omeprazole and infected with H.pylori infection are at increased risk of atrophic gastritis
Lamberts *et al*[55]	74 peptic ulcer patients	Omeprazole 48 mo	Median gastrin levels moderately increased after 3 mo of therapy and reached a plateau during maintenance treatment	Significant argyrophil cell hyperplasia

GERD: Gastroesophageal reflux disease; G-Gly: Glycine-extended gastrins; BE: Barrett's esophagus; PPI: Proton pump inhibitors.

role of different molecular forms of gastrin in Barrett's carcinogenesis are discussed below.

AMIDATED GASTRINS

Amidated gastrins are, in healthy subjects, the final and most abundant product in the gastrin biosynthesis pathway. Through the interaction with their receptor, CCKBR, amidated gastrins might be involved in the neoplastic progression of BE.

Amidated gastrins and CCKB receptor expression in BE

Barrett's mucosa expresses its own gastrin. Patients with BE show higher levels of amidated gastrins than healthy subjects[44], which might be a consequence of both PPI intake and autocrine gastrin production by Barrett's mucosa[36,50]. This autocrine gastrin production diminishes with the progression to dysplasia and EAC[44], and there is not a significant difference between serum gastrin levels in GERD and BE patients[42]. The expression of its receptor increases in response to inflammation. In almost all BE biopsy samples studied, CCKBR mRNA and protein are detected; while, they are only occasionally present in healthy tissue and their presence in EAC is unclear[4,36,50,70]. In addition, expression of the receptor increases cell proliferation[26,31,44]; therefore, CCKBR may have an important role in GERD ulcer healing[36,44].

Biological effects of amidated gastrins

In vitro studies determined that amidated gastrins may promote cell proliferation and migration of BE and EAC cells, and those effects are mediated through the interaction with CCKBR[4,26,36,44].

The effects of amidated gastrins are mediated, at least partially, by the induction of cyclooxygenases (COX)-2 expression and prostaglandins production[3]. COX are membrane proteins that catalyze the limiting step in the prostaglandin synthesis pathway. Prostaglandins are molecules that may promote carcinogenesis through stimulation of cell division, induction of angiogenesis, and inhibition of apoptosis[71,72]. As a consequence of the interaction between amidated gastrins and CCKBR, COX-2 is overexpressed in Barrett's mucosa, leading to an increase in prostaglandins synthesis and cell proliferation[44,73].

COX-2 overexpression is related to the development of other GI cancers, and the use of COX-2 inhibitors, such as non-steroidal anti-inflammatory drugs is associated with a reduction in the frequency and mortality of those tumors[74-78]. *In vitro* and *in vivo* studies have shown that COX-2 inhibitors decrease cell proliferation in BE[79] and reduce the risk of developing EAC[74], suggesting that COX-2 might be a key factor in Barrett's carcinogenesis.

COX-2 overexpression seems to be an early event in the neoplastic transformation of BE. Despite the great variability observed between subjects, COX-2 levels in biopsy samples are always higher in BE mucosa than in normal esophageal epithelium[44,50,73,80].

Thus, amidated gastrins might have a role in the neoplastic progression of BE rather than in its initial development since BE cells express higher levels of gastrin, CCKB receptor and also COX-2 than EAC[44] and normal esophageal cells.

GASTRIN SYNTHESIS INTERMEDIATES: PROGASTRIN, G-GLY AND CTFP

The biological activity of gastrin synthesis intermediates was unknown until 1994[81]. Experiments carried out to determine their ability to stimulate gastric acid secretion showed negligible or less potency than fully processed amidated forms[82,83]; therefore, those investigations concluded the intermediates were inactive peptides and focused mainly on the known bioactive forms. However, in the last few decades, numerous studies have demonstrated that these molecules are far from inactive precursors. Gastrin intermediates are secreted in higher proportions than their amidated forms in certain gastrin overexpression states[39,84]; thus, knowledge of their recently known biological effects has led to several studies on these intermediates in the last few decades. To date, most of these studies have been focused on CRC; although the relative abundance of these precursors in other tissues supports that it is necessary to extend research to other organs as well.

PROGASTRIN

PG is the first gastrin synthesis intermediate after signal peptide cleavage. A study demonstrated that PG levels in antral biopsies from patients undergoing PPI treatment were up to 6-fold higher than in untreated patients[39]; although, there are currently no studies showing PG plasma levels in response to PPI administration

Progastrin expression

Studies carried out on healthy colonic and CRC tissue have shown a higher proportion of products from the early stages of gastrin synthesis (PG above all) than those from later stages (G-Gly and amidated forms) in cancer samples[20,85]. Plasma PG levels, but not amidated gastrin, are elevated in CRC patients compared with healthy subjects and those with colonic polyps, suggesting a possible tumor origin for this PG and an incomplete processing of the peptide in tumor cells[22]. Other tumors, such as pancreatic, ovarian, and lung cancer, also overexpress PG[45,47,48].

Biological effects of PG

Progastrin may exert greater proliferative effects than amidated gastrins on normal and tumor cells (CRC, pancreatic) in culture[28,86] and also has an antiapoptotic effect[87]. *In vivo* studies using mice overexpressing both G17-NH_2 and PG showed increased colonic proliferation compared to wild-type control mice. At plasma concentrations similar to those observed in certain disease states, PG can act as a co-carcinogen and significantly increases the risk for colon carcinogenesis in response to azoxymethane[2,5].

PG has negligible affinity for the receptor for amidated gastrins (CCKBR) and its effects are mediated by a different receptor: ANX Ⅱ[27-30]. This receptor is not expressed on quiescent cells and it is necessary to mediate at least 50% of exogenous PG effects on intestinal cells and more than 80% of the effects of autocrine gastrins on CRC cells[29]. ANX Ⅱ is overexpressed in human CRC and may be related to a poor prognosis[88]. It is also overexpressed in a wide variety of tumors[88,89]. The mitogenic and antiapoptotic effects of PG seem to be mediated through activation of several signaling pathways including nuclear factor-κB (NF-κB), Src, Janus-activated kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3), extracellular signal-regulated kinase (ERK), mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt kinases[86,90].

G-GLY

G-Gly are some of the last gastrin processing intermediates. They result from the cleavage of the C-terminal arginyl residues of progastrin by carboxypeptidase E, before the amidation step.

G-Gly expression

As with other gastrin intermediates, there are few studies focused on assessing possible changes in plasma or tissue levels of G-Gly in different situations. It seems that PPIs don't significantly affect its levels because antral mucosa levels of G-Gly remain unaltered after treatment with PPIs[39]. In addition, CRC doesn't alter plasma G-Gly concentrations[22]; while, tumor biopsies and cell lines derived from CRC show higher levels than healthy tissue[20,22]. G-Gly levels are also increased in the gastric mucosa from patients with gastrinoma[91]. Outside the GI tract, only a small proportion of lung cancer cases analyzed showed G-Gly overexpression, which was inversely related to survival rates[45].

Biological effects of G-Gly

Even small variations in the levels of G-Gly may affect proliferation and apoptosis. G-Gly effects can be observed at concentrations at least one order of magnitude less than for amidated forms[33,92]. G-Gly can act as a growth factor for many cultured cells, including gastric, pancreatic, colonic cancer cells, and non-transformed cells[33,52,81,93,94]. It also decreases apoptosis in CRC and EAC[1,92] cells and increases migration in CRC cells[40]. In vivo experiments using transgenic mice overexpressing this intermediate demonstrated that higher G-Gly levels are related to colonic hyperproliferation, but were not

able to cause tumors alone[90]. Surprisingly these experiments showed higher bronchoalveolar cancer rates in the animals overexpressing the molecule[45,95].

The proliferative effects of G-Gly are dependant, at least partially, on COX-2 expression in EAC cells because the use of COX-2 inhibitors abolishes the proliferative effects of the molecule[52].

To date, the G-Gly receptor remains unknown but the majority of its effects seem to be mediated *via* a different receptor than CCKBR[1,33,96]. Although G-Gly has been shown to bind to ANX Ⅱ, no effects were observed[29]. The G-Gly interaction with a receptor distinct from CCKBR leads to JAK2/STAT3, Akt, NF-κB, PI3k, and ERK activation[52], increasing COX-2 expression. In contrast, the antiapoptotic effects of G-Gly occur independently from COX-2 expression[1].

CTFP

CTFP is the gastrin synthesis intermediate generated after cleavage of progastrin into its dibasic residues, generating G-Gly and the 6-amino acid CTFP. After the discoveries that the gastrin intermediates PG and G-Gly are present in normal antrum and certain tumors[20-22,48] and have effects enhancing cell proliferation and inhibiting apoptosis[5,28,29,33,52], several studies focused on those molecules. However, little attention has been paid to CTFP.

CTFP expression

In plasma and human antral extracts from healthy subjects, CFTP is the most abundant peptide (four-fold higher than the next most abundant peptide in antral samples and 30-fold higher in plasma). In CRC patients, CTFP levels are elevated in tumor mucosa and remain unaltered in plasma[32].

CTFP biological effects

CTFP effects have been tested on colonic and gastric cancer cell lines *in vitro*. CTFP showed higher potency in stimulating cell growth than G-Gly in colon tumor cells and a similar potency in gastric cancer cells. CTFP also stimulated cell migration in a non-transformed mouse gastric cell line and activated MAPK phosphorylation in colon cancer cells *via* a different receptor than CCKBR[32]. CTFP also exerts anti-apoptotic effects[97]. To date, only a few studies have focused on CTFP, but it has been determined that it is a biologically active molecule that is secreted in higher amounts than any other product of the gastrin gene. Thus, further experiments with this peptide should be carried out.

CONCLUSION

In summary, data derived from *in vitro* studies and animal models strongly suggest that high levels of gastrin may exert carcinogenic effects, on BE and colorectal epithelia, but also elsewhere. In addition, certain tumors produce their own gastrin, which might contribute to support tumor growth. However, it is currently not clear if high gastrin levels have the same effects in human beings. Most studies have been focused on amidated gastrins. Although, intermediates of gastrin synthesis can exert even greater carcinogenic effects than the amidated forms and in certain situations they become the most abundant forms of gastrin. Therefore, more studies evaluating these molecules are needed to elucidate the potential role of gastrins in human carcinogenesis.

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S- Editor Gou SX **L- Editor** A **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v18.i45.6571

World J Gastroenterol 2012 December 7; 18(45): 6571-6576
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Relatedness of *Helicobacter pylori* populations to gastric carcinogenesis

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Received: June 1, 2012 Revised: August 17, 2012
Accepted: August 25, 2012
Published online: December 7, 2012

Abstract

Helicobacter pylori (*H. pylori*) is a Gram-negative bacterium that infects half of the human population. The infection is associated with chronic inflammation of the gastric mucosa and peptic ulcers. It is also a major risk factor for gastric cancer. Phylogenetic analysis of global strains reveals there are seven populations of *H. pylori*, including hpAfrica1, hpAfrica2, hpEastAsia, hpEurope, hpNEAfrica, hpAsia2 and hpSahul. These populations are consistent with their geographical origins, and possibly result from geographical separation of the bacterium leading to reduced bacterial recombination in some populations. For each population, *H. pylori* has evolved to possess genomic contents distinguishable from others. The hpEurope population is distinct in that it has the largest genome of 1.65 mbp on average, and the highest number of coding sequences. This confers its competitive advantage over other populations but at the cost of a lower infection rate. The large genomic size could be a cause of the frequent occurrence of the deletion of the *cag* pathogenicity island in *H. pylori* strains from hpEurope. The incidence of gastric cancer varies among different geographical regions. This can be attributed in part to different rates of infection of *H. pylori*. Recent studies found that different populations of *H. pylori* vary in their carcinogenic potential and contribute to the variation in incidence of gastric cancer among geographical regions. This could be related to the ancestral origin of *H. pylori*. Further studies are indicated to investigate the bacterial factors contributing to differential virulence and their influence on the clinical features in infected individuals.

Key words: *Helicobacter pylori*; Population genetics; Gastric cancer; Virulence; Genome

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Dong QJ, Zhan SH, Wang LL, Xin YN, Jiang M, Xuan SY. Relatedness of *Helicobacter pylori* populations to gastric carcinogenesis. *World J Gastroenterol* 2012; 18(45): 6571-6576 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i45/6571.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i45.6571

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative bacterium which colonizes the human stomach. As a pathogen, *H. pylori* induces inflammation of the gastric mucosa[1]. It plays a causal role in the ulceration and recurrence of peptic ulcer[2]. Eradication of the bacterium heals ulcers and prevents recurrence of the disease. The infection is also associated with an increased risk of gastric cancer[3,4].

The incidence of gastric cancer shows geographical variation. This is attributed in part to the difference in the prevalence of the *H. pylori* infection among geo-

graphical regions. In Africa and South Asia, however, the incidence of gastric cancer in these areas is much lower than in other countries in spite of the high prevalence of the *H. pylori* infection[5]. Such a disparity has also been found in other local regions[6]. Analysis of global strains reveals seven populations of *H. pylori* that are consistent with their geographical origin[7-10]. These current populations derive from six ancestral populations[7]. It appears that the ancestry, genomic contents and carcinogenic potentials are diversified among *H. pylori* populations. Studies at a population level have improved our understanding of gastric carcinogenesis associated with the *H. pylori* infection.

GENETIC DIVERSITY AND POPULATIONS OF *H. PYLORI*

There are three types of bacterial population structure: clonal, panmictic and endemic[11]. If intra-species or inter-species recombination is rare, the genetic diversity of a bacterial species predominantly comes from evolution of the ancestry. This species has a clonal population structure. In a species with high frequency of recombination, introduction of foreign gene fragments into the genome occurs frequently in the evolution history. As foreign genes have a different evolution history, the evolution speed of individual genes is different. In this case, the species possess a panmictic structure. For a bacterial species with a panmictic structure, a temporal clonal structure may occur if it rapidly spread among naïve hosts. In this situation, a bacterial species has an endemic structure.

H. pylori shows great inter-strain variation in genetic content[12]. None of the individual strains is identical as demonstrated by multiple fingerprinting methods[13,14]. Sequence divergence is the main cause of this variation. Comparison of two sequenced genomes revealed occurrence of substantial silent mutation in the genetic loci[15]. A number of mechanisms are involved in the generation of the sequence variation: *H. pylori* shows a higher mutation rate than *Escheria coli*[16]. Approximately a quarter of strains possess a mutator-like phenotype. This is attributed to the lack of a functional DNA repair system[16,17] and error-prone DNA polymerase in *H. pylori*[18]. Recombination in *H. pylori* is more frequent than in any other organism studied to date[19]. Foreign DNA from the same species or phage has been found in the bacterium[15]. Strand slippage mispairing is another mechanism responsible for genetic diversity. A number of homopolymeric tracts and dinucleotide repeat regions are present in the *H. pylori* genome[20,21], which may cause replication error and subsequently sequence variation. *H. pylori* has a specialized type Ⅳ system for uptake of foreign DNA from the same species or other species[22]. Foreign DNA fragments are subsequently integrated into the genome by recombination. A high frequency of recombination and a high mutation rate in *H. pylori* result in a panmictic structure of the bacterium[23].

Recombination is a rare genetic event in house-keeping genes. Phylogenetic analysis of highly conserved house-keeping genes (*atpA*, *efp*, *mutY*, *ppa*, *trpC*, *ureI* and *yphC*) has then been used to study populations of *H. pylori*[24]. Examination of global strains of *H. pylori* reveals that it has seven populations: hpAfrica1, hpAfrica2, hpEastAsia, hpEurope, hpNEAfrica, hpAsia2 and hpSahul[7-10,25,26]. Some populations could be further divided into subpopulations. For hpEastAsia, there are three subpopulations including hspEAsia, hspAmerind and hspMaori[27], while hpAfrica1 is split into hspWAfrica and hspSAfrica[28]. These populations and subpopulations reflect not only their geographical origin but also ethnic groups of their hosts. Spatial separation reduces bacterial recombination between different geographical regions. A weakly clonal population may thus be generated in strains from a particular geographical region[29]. *H. pylori* spreads mainly through a mode of family transmission[30,31], leading to a reduced chance of recombination between strains from different ethnic groups. Therefore, strains from different ethnic groups could be distinguished in the phylogenetic analysis. This is one of the features that allows phylogenetic analysis of *H. pylori* to be used to trace the history of human migration.

GENOMIC DIVERGENCE BETWEEN *H. PYLORI* POPULATIONS

H. pylori has been accompanying human hosts for more than 58 000 years[10]. The genome of *H. pylori* is thus shaped by its human hosts due to the long co-existence[32]. *H. pylori* strains differ in their affinity to bind blood group antigens expressed in the gastric mucosa[33]. Strains from Europe bind all three blood group antigens, while Amerindian strains have higher affinity for O blood antigen as this antigen is predominant in Amerindians[32]. Therefore, the genomic content of *H. pylori* may vary in different populations.

To date, a number of strains of *H. pylori* have been sequenced[15,34-37]. Of these, the origin and other information of 30 strains are publicly available. These include 14 strains from hpEastAsia (7 from hspEasia and 7 from hspAmerind subpopulations, respectively), 10 from hpEurope, 5 from hpAfrica1 and 1 from hpAfrica2[38-41]. All, except for strain B38 from hpEurope, possess *cagA* and the *cag* pathogenicity island (*cag* PAI). The genomic size of *cagA*-positive *H. pylori* ranges from approximately 1.55 mbp to 1.71 mbp with an average of 1.61 mbp. For *cagA*-negative strains, their genome is generally smaller because of the lack of the *cag* pathogenicity island of about 40 kbp. We analyzed the average genomic size of *cagA*-positive *H. pylori* strains from different populations[42-47]. The average genomic size of strains from hpEurope is approximately 1.65 mbp, which is significantly larger than that from hpEastAsia (1.60 mbp, $P < 0.05$) or hpAfrica1 (1.60 mbp). Consistent with this, strains of hpEurope have the highest number of coding sequences. There was a statistically significant difference between hpEurope and

hpEastAsia in the average genomic size and number of CDS. The size of bacterial genomes is primarily determined by two counteracting processes: the gain of new genes by gene duplication or by horizontal gene transfer; and the decay of non-essential genes[15]. Both of these processes have been observed in *H. pylori*. Recombination, conjugation, insertion elements, mutation and slipped-strain replication lead to gene gain or loss[34]. They may be involved in the variability of genomic size among *H. pylori* of different populations.

For bacteria, a larger genome requires more metabolic activities and consumes more energy[48,49]. Therefore bacteria containing a larger genome may have a lowered capacity of growth, reducing its competitive ability with other bacteria in the same ecological niche. This leads to a decreased spread of bacteria. It is well known that *H. pylori* is less prevalent in Western countries than in other parts of the world[50]. Hygiene, economical incomes and social status have been suggested to contribute to this differential prevalence[51]. It is arguable that a larger genome of *H. pylori* strains in Western countries may also contribute to the low prevalence of the infection in this region.

Comparison of the genomic content of *H. pylori* from different populations revealed differences in the compositions of outer membrane proteins and central metabolism[39]. Compared with hpEurope, strains from hpEastAsia tend to have fewer genes of these two categories. There are a total of 12 genes in *H. pylori* involved in molybdenum metabolism, including those encoding proteins for molybdenum transport and cofactor synthesis and a molybdenum-containing enzyme. A massive decay of molybdenum-related genes occurs in strains from hpEastAsia. At least five genes are fragmented due to mutations. The molybdenum-containing enzyme functions in electron transfer and responses to oxidative and acid stress[52]. It is probable that in hpEastAsia populations, *H. pylori* use alternative pathways for the purpose[39]. Outer membrane proteins consist of several paralog families interacting with the human host[53,54]. In the hpEastAsia population, there is a tendency for a reduced number of these proteins resulting from mutations and recombination. Therefore it appears that *H. pylori* from hpEastAsia have evolved to possess a reduced genome.

The *cag* PAI is a 40-kb DNA fragment which contains 27 to 31 genes flanked by 31-bp direct repeats[55]. It encodes CagA, the major virulence determinant of *H. pylori* and components of a type Ⅳ secretion system[56,57]. The latter translocates CagA into host cells[58]. Once inside the host cells, CagA binds to a number of host cell proteins disrupting intracellular signaling systems *via* tyrosine phosphorylation-dependent or -independent pathways[59]. This causes elongation and loss of polarity of host cells, promoting proliferation and inflammation. The presence of the *cag* PAI in *H. pylori* is associated with increased risk of severe gastritis, atrophic gastritis, and distal gastric cancer compared with strains that lack the *cag* island[60-62].

A marked difference lies between hpEurope and hpEastAsia in the prevalence of strains possessing the *cag* PAI. Approximately 60% to 70% of Western *H. pylori* strains express CagA[61,63], indicating the presence of the *cag* PAI. In East Asia, however, almost 100% of strains possess the *cag* PAI irrespective of pathology[64,65]. It is believed that the *cag* PAI is deleted in Western strains resulted from recombination between the repeats flanking the island[66]. This results in a reduced genomic size by approximately 40 kbp. In addition, it has been demonstrated that the prevalence of strains with an intact cag PAI is the lowest in Western countries[67]. As described above, strains from hpEurope are coincidently 40 kbp larger than the average genomic size of *H. pylori*. Thus, the occurrence of *cagA*-negative strains in hpEurope is probably due to the evolution of the bacterium towards a smaller genome.

VARIATION IN THE CARCINOGENIC POTENTIAL OF *H. PYLORI* POPULATIONS

The incidence of gastric cancer varies in different geographical regions. It is higher in East Asian countries than in any other countries when age-standardized rates are considered[68]. In some countries of West Africa and South America, there is also an increased incidence of gastric cancer[69,70]. The geographic difference in the incidence of gastric cancer can be attributed partially to the difference in the prevalence of *H. pylori* infection[71]. A high prevalence of virulent strains of *H. pylori* is another contributing factor to the high incidence of gastric cancer in East Asia. Virulent strains possess the *cag* PAI and express *Vac*A. There is, however, a disparity between the prevalence of *H. pylori* or virulent strains and the incidence of gastric cancer. In Linqu County, China, the incidence of gastric cancer is extremely high, while in its neighboring county Cangshan the incidence is very low[72,73]. The rate of *H. pylori* infection and the proportion of virulent strains in these two counties, however, show no significant difference[74]. Similar results have been found when comparing two regions in Mexico with contrasting incidence of gastric cancer[75]. These results indicate differential incidence of gastric cancer among different geographical regions is attributable to other bacterial factors.

To explore other bacterial factors related to carcinogenesis, the phylogeographical origin of *H. pylori* has been investigated[76]. In the Andean mountain region of Colombia, habitants have a high incidence of gastric cancer (150 per 100 000 people per year)[77,78], while habitants in the coastal line 200 kilometers away, have a very low incidence of gastric cancer (6/100 000)[77,78]. The prevalence of the infection and virulent strains of *H. pylori* in these two regions are similar[75]. All *H. pylori* strains isolated from the Andean region, however, are from hpEurope, in contrast to strains isolated from the coastal line which are mainly from hpAfrica1[76]. Furthermore, strains from the Andean region caused more severe mucosal inflammation and more DNA damage in epithelial cells. This suggests that strains of hpEurope probably have an

increased carcinogenic potential compared with those of hpAfrica1[76]. Ancestral origin of the bacterium could be an important factor contributing to gastric carcinogenesis. This conclusion is further supported from a study conducted in Malaysia[7]. There are three ethnic groups in the country: Malay, Indian and Chinese. The infection rate of *H. pylori* in Malays is lower than that in Indian and Chinese subjects[79]. The incidence of gastric cancer, however, is similar in Malays and Indians, but is much lower than in the Chinese[80]. Analysis of the ancestral origin of *H. pylori* found that strains isolated from both Malay and Indian subjects belonged to hpAsia2, whereas those isolated from Chinese subjects belonged to hpEastAsia. This suggests a different potential for carcinogenesis between hpAsia2 and hpEastAsia. *H. pylori* populations generally reflect the geographical regions from which they are isolated. Differences in the incidence of gastric cancer among geographical regions could be in part attributed to different populations of *H. pylori*. Further study is required to investigate other bacterial factors involved in the carcinogenesis.

CONCLUSION

In summary, geographical separation reduces the frequency of recombination between *H. pylori* strains from a local area and those from outside. This leads to the formation of a clonal population structure of *H. pylori* in the local area. Thus, populations of *H. pylori* could be identified through examination of global strains. For each population, *H. pylori* have experienced relatively separate evolution processes, resulting in genomic diversity and differential potential for carcinogenesis. Further study to characterize these differences may help elucidate mechanisms involved in the development of gastric cancer induced by *H. pylori*.

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S- Editor Gou SX **L- Editor** Cant MR **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v18.i45.6577

World J Gastroenterol 2012 December 7; 18(45): 6577-6586
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Alterations in the human epidermal growth factor receptor 2-phosphatidylinositol 3-kinase-v-Akt pathway in gastric cancer

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Supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, to Yamamoto H and Shinomura Y
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Received: June 18, 2012 Revised: August 21, 2012
Accepted: August 25, 2012
Published online: December 7, 2012

Abstract

AIM: To investigate human epidermal growth factor receptor 2 (HER2)-phosphatidylinositol 3-kinase (PI3K)-v-Akt murine thymoma viral oncogene homolog signaling pathway.

METHODS: We analyzed 231 formalin-fixed, paraffin-embedded gastric cancer tissue specimens from Japanese patients who had undergone surgical treatment. The patients' age, sex, tumor location, depth of invasion, pathological type, lymph node metastasis, and pathological stage were determined by a review of the medical records. Expression of HER2 was analyzed by immunohistochemistry (IHC) using the HercepTest™ kit. Standard criteria for HER2 positivity (0, 1+, 2+, and 3+) were used. Tumors that scored 3+ were considered HER2-positive. Expression of phospho Akt (pAkt) was also analyzed by IHC. Tumors were considered pAkt-positive when the percentage of positive tumor cells was 10% or more. PI3K, catalytic, alpha polypeptide (PIK3CA) mutations in exons 1, 9 and 20 were analyzed by pyrosequencing. Epstein-Barr virus (EBV) infection was analyzed by *in situ* hybridization targeting EBV-encoded small RNA (EBER) with an EBER-RNA probe. Microsatellite instability (MSI) was analyzed by polymerase chain reaction using the mononucleotide markers BAT25 and BAT26.

RESULTS: HER2 expression levels of 0, 1+, 2+ and 3+ were found in 167 (72%), 32 (14%), 12 (5%) and 20 (8.7%) samples, respectively. HER2 overexpression (IHC 3+) significantly correlated with intestinal histological type (15/20 *vs* 98 /205, $P = 0.05$). PIK3CA mutations were present in 20 cases (8.7%) and significantly correlated with MSI (10/20 *vs* 9/211, $P < 0.01$).

The mutation frequency was high (21%) in T4 cancers and very low (6%) in T2 cancers. Mutations in exons 1, 9 and 20 were detected in 5 (2%), 9 (4%) and 7 (3%) cases, respectively. Two new types of PIK3CA mutation, R88Q and R108H, were found in exon1. All PIK3CA mutations were heterozygous missense single-base substitutions, the most common being H1047R (6/20, 30%) in exon20. Eighteen cancers (8%) were EBV-positive and this positivity significantly correlated with a diffuse histological type (13/18 *vs* 93/198, P = 0.04). There were 7 cases of lymphoepithelioma-like carcinomas (LELC) and 6 of those cases were EBV-positive (percent/EBV: 6/18, 33%; percent/all LELC: 6/7, 86%). pAkt expression was positive in 119 (53%) cases but showed no correlation with clinicopathological characteristics. pAkt expression was significantly correlated with HER2 overexpression (16/20 *vs* 103/211, P < 0.01) but not with PIK3CA mutations (12/20 *vs* 107/211, P = 0.37) or EBV infection (8/18 *vs* 103/211, P = 0.69). The frequency of pAkt expression was higher in cancers with exon20 mutations (100%) than in those with exon1 (40%) or exon9 (56%) mutations. One case showed both HER2 overexpression and EBV infection and 3 cases showed both PIK3CA mutations and EBV infection. However, no cases showed both PIK3CA mutations and HER2 overexpression. One EBV-positive cancer with PIK3CA mutation (H1047R) was MSI-positive. Three of these 4 cases were positive for pAkt expression. In survival analysis, pAkt expression significantly correlated with a poor prognosis (hazard ratio 1.75; 95%CI: 1.12-2.80, P = 0.02).

CONCLUSION: HER2 expression, PIK3CA mutations and EBV infection in gastric cancer were characterized. pAkt expression significantly correlates with HER2 expression and with a poor prognosis.

Key words: Human epidermal growth factor receptor 2; Phosphatidylinositol 3-kinase; Catalytic; Alpha polypeptide; Epstein-Barr virus; Akt; Gastric cancer

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INTRODUCTION

Gastric cancer is one of the most common cancer types and the second leading cause of cancer-related deaths worldwide[1]. Genetic and epigenetic alterations play important roles in the development and progression of these tumors[1,2]. Considerable attention has been given to the potential role of the phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathway in gastric cancer[3,4]. Various alterations, such as activation of growth factor receptors, PI3K, catalytic, alpha polypeptide (PIK3CA) mutations and inactivation of phosphatase and tensin homolog (PTEN) lead to activation of the PI3K-Akt signaling pathway. With regards to growth factor receptors, there is growing evidence that human epidermal growth factor receptor 2 (HER2) is a key driver of tumorigenesis and an important biomarker in gastric cancer. The amplification or overexpression of HER2 is observed in 7%-34% of these cases[5-9].

PIK3CA is mutated in a wide variety of human tumor types[10,11], including gastric cancers[12-15]. Activating mutations in this gene up-regulate the PI3K-Akt signaling pathway, making it a potentially useful therapeutic target. For example, oncogenic mutations of PIK3CA reportedly render breast cancers more resistant to treatment with the anti-HER2 receptor antibody trastuzumab[16]. Thus, this signaling pathway is thought to be one of the mechanisms underlying resistance to trastuzumab. Trastuzumab has recently been approved for treatment of advanced gastric cancers[5,6].

Pyrosequencing-based methods facilitate the identification of low-frequency tumor mutations and allow a more accurate assessment of tumor mutation burden[17]. PIK3CA mutations have been detected in 4%-25% of gastric cancers[12-15]. However, in most previous studies, only exons 9 and 20 hot spot mutations in PIK3CA were analyzed by DNA sequencing. Moreover, the association between HER2 expression and PIK3CA mutations in gastric cancer has not been reported.

A significant correlation has been found between Epstein-Barr virus (EBV) and the methylation of multiple genes in gastric cancers[18-20]. EBV infection reportedly induces PTEN expression loss through CpG island methylation of its promoter, leading to activation of the PI3K-Akt signaling pathway, in EBV-associated gastric cancer[21].

The aim of our present study was to systematically characterize HER2 expression, PIK3CA mutations, and EBV infection, all of which are involved in the PI3K-Akt signaling pathway, in a large cohort of gastric cancers (n = 231). We wished to determine the prevalence of each of these factors with a high precision and thereby correlate them with clinicopathological and molecular features of gastric lesions, including microsatellite instability (MSI) and phospho Akt (pAkt) expression.

MATERIALS AND METHODS

Tissue samples

A total of 231 formalin-fixed, paraffin-embedded (FFPE) gastric cancer tissue specimens from Japanese patients who had undergone surgical treatment was analyzed in

Table 1 Clinicopathological characteristics of patients with gastric cancer

Variables (n = 231)		n (%)
Sex	Male	157 (68)
	Female	74 (32)
Age (yr)	Median (range)	71 (25-91)
Location	Cardias	82 (35)
	Body	62 (27)
	Antrum	83 (36)
	Unknown	4 (2)
Depth of invasion	T2	125 (54)
	T3	92 (40)
	T4	14 (6)
Lymph node metastasis	N0	65 (28)
	N+	158 (68)
	N1	73 (32)
	N2	56 (24)
	N3	29 (13)
	Unknown	8 (3)
Stage	Ⅰ B	49 (21)
	Ⅱ	45 (19)
	ⅢA + ⅢB	82 (35)
	Ⅳ	51 (22)
	Unknown	4 (2)
Lauren histotype	Intestinal	113 (49)
	Diffuse	112 (48)
	Others	6 (3)

this study. The patients' age, sex, tumor location, depth of invasion, pathological type, lymph node metastasis, and pathological stage were determined by a review of their medical records. Clinicopathological findings were determined according to the criteria of the Japanese Research Society for Gastric Cancer (Table 1). Our institutional review committee approved the study.

Immunohistochemistry

HER2 expression was analyzed using the HercepTest™ kit (DAKO, Carpinteria, CA) by manual sample processing in accordance with the manufacturer's instructions. Standard criteria for HER2 positivity (0, 1+, 2+ and 3+) were used. Tumors that scored 3+ were considered HER2-positive. For the immunohistochemical analysis of pAkt, FFPE specimens were processed using SignalStain Boost Detection Reagent (Cell Signaling Technology, Beverly, MA). Briefly, 5-μm-thick sections were dewaxed in xylene, rehydrated in ethanol, and heated with target retrieval solution (DAKO) in an autoclave for antigen retrieval. Endogenous peroxidase was blocked by incubation with 0.3% hydrogen peroxide in methanol for 10 min. The tissue sections were then washed twice with tris-buffered saline (TBS) and preblocked with 10% goat serum in TBS for 60 min. After washing with TBS, the sections were incubated with an anti-phospho-Akt (Ser473) polyclonal antibody (D9E, Cell Signaling Technology) at a dilution of 1:100 for 30 h at 4 ℃. The sections were washed three times in TBS and incubated with SignalStain Boost Detection Reagent for 45 min. After three further washes in TBS, a diamino-benzidine tetrahydrochloride working solution was applied. Finally, the sections were counterstained with hematoxylin. Tumors were considered pAkt-positive when the percentage of positive tumor cells was 10% or more[22]. Only clear staining of the tumor cell nucleus and/or cytoplasm was considered positive.

Mutation analysis of the PIK3CA gene by pyrosequencing

Genomic DNA was extracted from tumor specimens and mutations in exon9 and exon20 of the *PIK3CA* gene were analyzed by pyrosequencing as described previously[23,24]. We also developed a pyrosequencing assay to detect PIK-3CA exon1 mutations using the primer sets exon1-RS1 (5'-GGGAAGAATTTTTTGATGAAACA-3' for the biotinylated forward primer and 5'-GGTTGCCTACT-GGTTCAATTACTT-3' for the reverse primer) and exon1-RS2 (5'-CGGCTTTTTCAACCCTTTTT-3' for the forward primer and 5'-ATTTCTCGATTGAG-GATCTTTTCT-3' for the biotinylated reverse primer). Each polymerase chain reaction (PCR) mix contained the forward and reverse primers (each 10 μmol/L), a 25 mmol/L dNTP mix with dUTP, 75 mmol/L $MgCl_2$, 1 × PCR buffer, 1.0 U of exTaq, and 2 μL of template DNA in a total volume of 25 μL. PCR conditions were as follows: initial denaturing at 95 ℃ for 5 min; 50 cycles of 94 ℃ for 20 s, 50 ℃ for 20 s and 74 ℃ for 40 s; and a final extension at 72 ℃ for 1 min. The PCR products (each 25 μL) were sequenced using the PyroMark kit and Pyrosequencing PSQ96 HS System (Qiagen, Valencia, CA).

In situ hybridization for EBER1

The presence of EBV in the carcinoma tissues was evaluated by *in situ* hybridization (ISH) targeting of EBV-encoded small RNA (EBER-ISH) with an EBER-RNA probe (Dako Cytomation).

Microsatellite instability analysis

MSI was analyzed by PCR using the mononucleotide markers (BAT25 and BAT26). Based on the number of markers showing instability per tumor sample, cancers were divided into two groups; those with one or more of the two markers displaying MSI and those with no instability (microsatellite stable).

Statistical analysis

For all statistical analysis, the JMP program was used. All P values were two-sided and statistical significance was set at $P \leqslant 0.05$. For categorical data, the χ^2 test was used. For survival analysis, Kaplan-Meier method and log-rank test were used. For analysis of cancer-specific mortality, we excluded surgery-related deaths (deaths within one month of surgery).

RESULTS

HER2 expression in gastric cancer tissues

HER2 expression levels of 0, 1+, 2+ and 3+ were found in 167 (72%), 32 (14%), 12 (5%) and 20 (8.7%) samples, respectively (Figure 1). HER2 overexpression (IHC 3+) significantly correlated with intestinal histological type

Table 2 Clinicopathological characteristics of patients with gastric cancer based on human epidermal growth factor receptor 2 expression, phosphatidylinositol 3-kinase, catalytic, alpha polypeptide mutations and Epstein-Barr virus infection *n* (%)

		HER2			PIK3CA			EBV		
		Positive (*n* = 20)	Negative (*n* = 211)	*P* value	Mutation (*n* = 20)	Wild type (*n* = 211)	*P* value	Positive (*n* = 18)	Negative (*n* = 204)	*P* value
Sex	Male	15 (75)	142 (67)	0.48	13 (65)	144 (68)	0.77	14 (78)	138 (68)	0.38
	Female	5 (25)	69 (33)		7 (35)	70 (32)		4 (22)	66 (32)	
Age	Median	69 (50-84)	71 (25-91)	0.26	71 (25-85)	70 (38-91)	0.40	72 (48-90)	70 (38-91)	0.41
Location	Cardias	10 (50)	72 (34)	0.49	5 (25)	77 (36)	0.31	8 (44)	73 (36)	0.70
	Body	5 (25)	57 (27)		4 (20)	58 (27)		5 (28)	55 (27)	
	Antrum	5 (25)	78 (37)		10 (50)	73 (35)		5 (28)	75 (37)	
	Unknown	0	4 (2)		1 (5)	2 (1)		0	1 (0)	
Depth	T2	12 (60)	113 (54)	0.48	8 (40)	117 (55)	0.15	12 (67)	106 (52)	0.35
	T3	8 (40)	84 (40)		9 (45)	83 (39)		6 (33)	85 (42)	
	T4	0	14 (6)		3 (15)	11 (5)		0	13 (6)	
L/N meta	N0	5 (25)	60 (28)	0.71	4 (20)	61 (29)	0.37	3 (17)	57 (28)	0.28
	N+	14 (70)	144 (68)		16 (80)	142 (67)		14 (77)	140 (69)	
	N1	5 (25)	68 (32)		8 (40)	65 (31)		8 (44)	63 (31)	
	N2	6 (30)	50 (24)		6 (30)	50 (24)		2 (11)	53 (26)	
	N3	3 (15)	26 (12)		2 (10)	27 (13)		4 (22)	24 (12)	
	Unknown	1 (5)	7 (3)		0	8 (4)		1 (6)	7 (3)	
Stage	I	5 (25)	44 (21)	0.89	1 (5)	48 (23)	0.14	3 (17)	41 (20)	0.98
	II	3 (15)	42 (20)		7 (35)	38 (18)		4 (22)	39 (19)	
	III	6 (30)	76 (36)		8 (40)	74 (35)		6 (33)	75 (37)	
	IV	5 (25)	46 (22)		4 (20)	47 (22)		4 (22)	46 (23)	
	Unknown	1 (5)	3 (1)		0	4 (2)		1 (6)	3 (1)	
Lauren	Intestinal	15 (75)	98 (46)	0.05	14 (70)	99 (47)	0.13	5 (28)	105 (51)	0.04
histotype	Diffuse	5 (25)	107 (51)		6 (30)	106 (50)		13 (72)	93 (46)	
	LELC	0	6 (3)		2 (10)	4 (2)		5 (28)	0	
	Others	0	6 (3)		0	6 (3)		0	6 (3)	
MSI		2 (10)	28 (13)	0.72	10 (50)	20 (9)	< 0.01	1 (6)	26 (13)	0.36
pAkt		16 (84)	103 (51)	< 0.01	12 (63)	107 (53)	0.37	8 (47)	103 (52)	0.69
3 yr OS (%)		29.4	59.2	0.24	57.3	56.8	0.59	57.4	57.3	0.98

MSI: Microsatellite instability; LELC: Lymphoepithelioma-like carcinoma; HER2: Human epidermal growth factor receptor 2; PIK3CA: Phosphatidylinositol 3-kinase, catalytic, alpha polypeptide mutations; EBV: Epstein-Barr virus; pAkt: Phospho Akt; OS: Overall survival.

Table 3 Frequencies of phosphatidylinositol 3-kinase, catalytic, alpha polypeptide mutations detected in gastric cancer tissues

	Mutation	Overall frequency	Percent/ total cases	Percent/ mutated cases	Microsatellite instability
Exon1	R88Q	1	0.4	5	1
	R108H	4	1.7	20	1
	Total	5	2.2		2
Exon9	E542K	5	2.2	25	1
	E545K	2	0.9	10	1
	E545G	2	0.9	10	1
	Total	9	4.0		3
Exon20	H1047Y	1	0.4	5	1
	H1047R	6	2.6	30	4
	Total	7	3.0		5

(15/20 *vs* 98/205, $P = 0.05$, Table 2). Three-year survival rates were 29% in patients with HER2 overexpression and 59% in cases without HER2 overexpression, respectively [hazard ratio (HR) 1.73; 95%CI: 0.87-3.14, $P = 0.24$].

Mutations of the PIK3CA gene in gastric cancer tissues

PIK3CA mutations were present in 20 cases (8.7%) (Table 2 and Figure 2). The mutation frequency was high (21%) in T4 cancers and low (6%) in T2 cancers. Mutations in exons 1, 9 and 20 of PIK3CA were detected in 5 (2%), 9 (4%) and 7 (3%) cases, respectively (Table 3). One case had multiple PIK3CA mutations (R108H and E542K). The exon20/exon9 prevalence ratio was 0.78 (7/9). Two new types of PIK3CA mutations, R88Q and R108H, were detected in exon1. All mutations were heterozygous missense single-base substitutions and the most common mutation was H1047R (6/20; 30%) in exon20. PIK3CA mutations were also found to significantly correlate with MSI (10/20 *vs* 9/211, $P < 0.01$) but not with other clinicopathological characteristics. The three-year survival rates were 57% in patients with PIK3CA mutations and 57% in cases without PIK3CA mutations, respectively (HR 1.37; 95%CI: 0.68-3.26, $P = 0.59$).

EBV infection

Eighteen samples in our cohort (8%) were EBV-positive and this positivity significantly correlated with diffuse histological type (13/18 *vs* 93/198, $P = 0.04$) (Table 2 and Figure 3). There were 7 cases of LELC and 6 of those cases were EBV-positive (percent/EBV: 6/18, 33%; percent/all LELC: 6/7, 86%). The three-year survival rates were 57% in patients with EBV infection and 57% in those without EBV infection (HR 0.81; 95%CI:

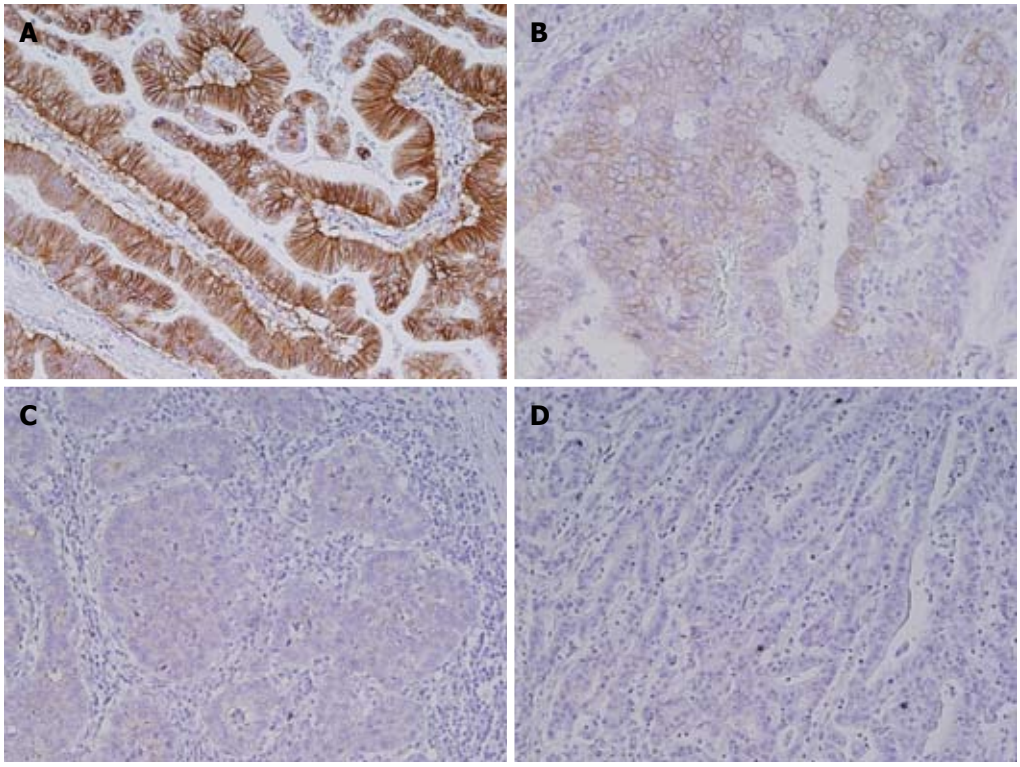

Figure 1 Immunohistochemical analysis of human epidermal growth factor receptor 2 in gastric cancer tissues. A: Human epidermal growth factor receptor 2 (HER2) 3+; B: HER2 2+; C: HER2 1+; D: HER2 0. Original magnification, ×200.

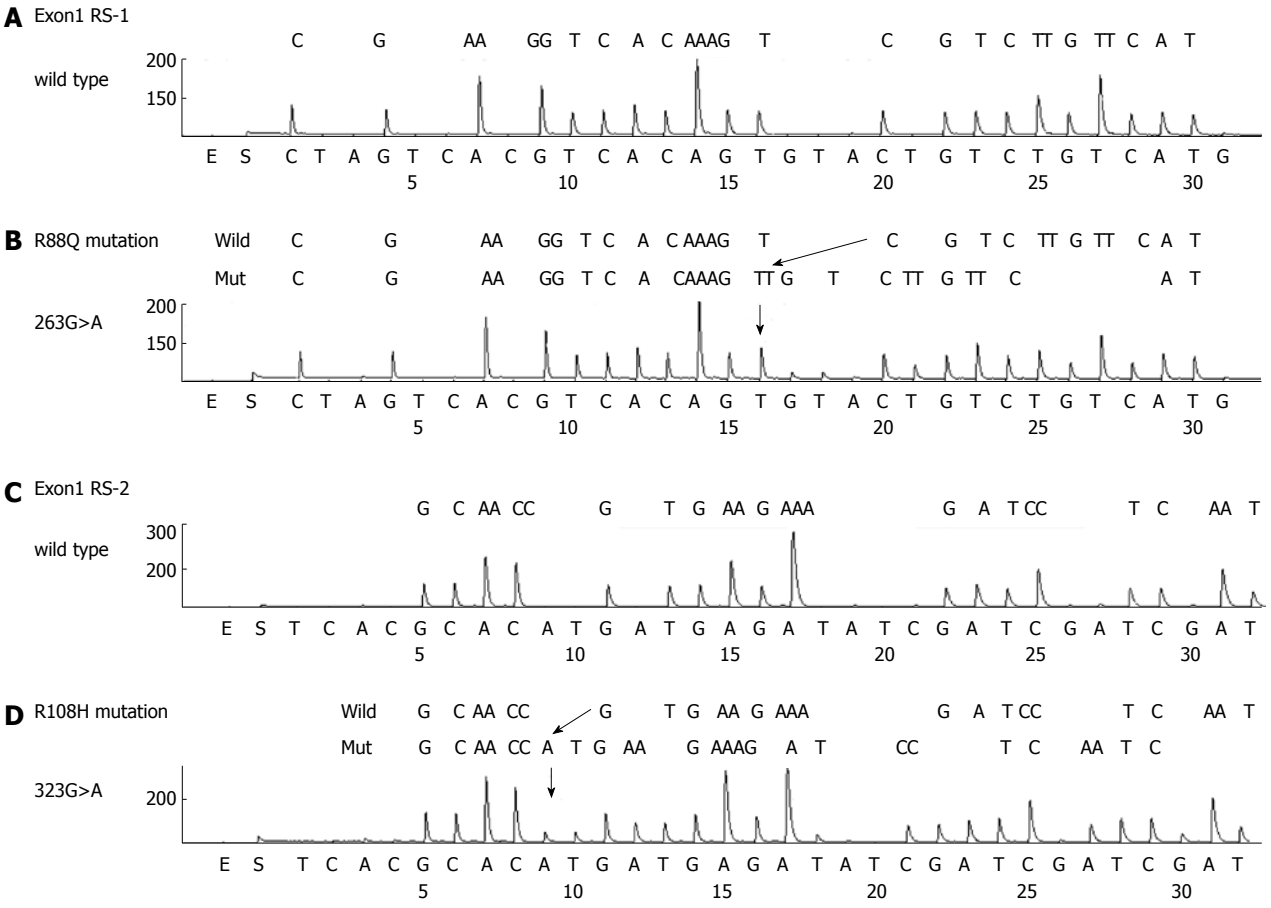

Figure 2 Phosphatidylinositol 3-kinase, catalytic, alpha polypeptide mutations detected by pyrosequencing in gastric cancer tissues. A: Exon1 RS1 wild type; B: 263G>A (R88Q) mutation; C: Exon1 RS2 wild type; D: 323G>A (R108H) mutation.

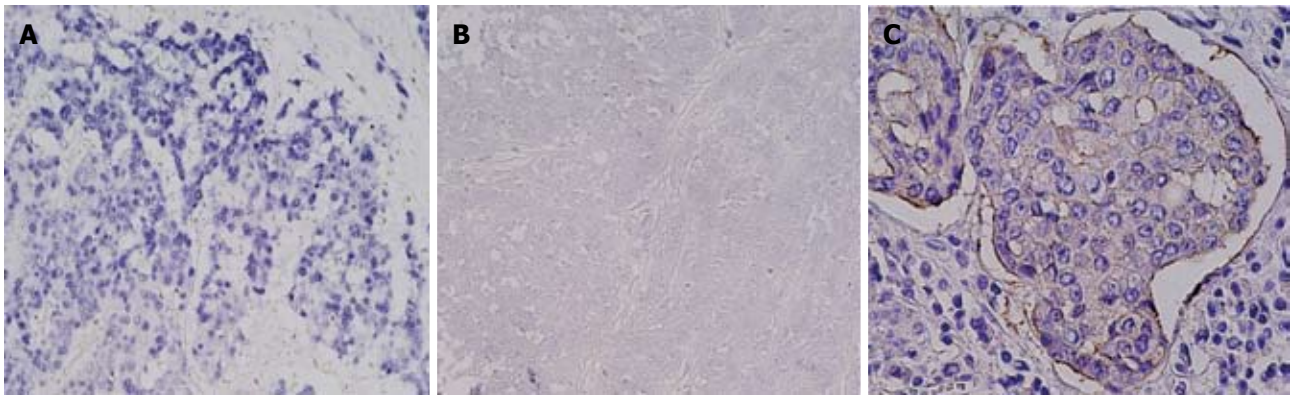

Figure 3 *In situ* hybridization analysis of Epstein-Barr virus-encoded small RNA-1 and human epidermal growth factor receptor 2 immunohistochemical expression in gastric cancer tissues. A: Gastric adenocarcinoma positive for Epstein-Barr virus-encoded small RNA-1 (EBER-1); B: Gastric adenocarcinoma negative for EBER-1; C: Immunohistochemical analysis of human epidermal growth factor receptor 2 (HER2) in an Epstein-Barr virus-positive and HER2-positive case. Original magnification, ×200.

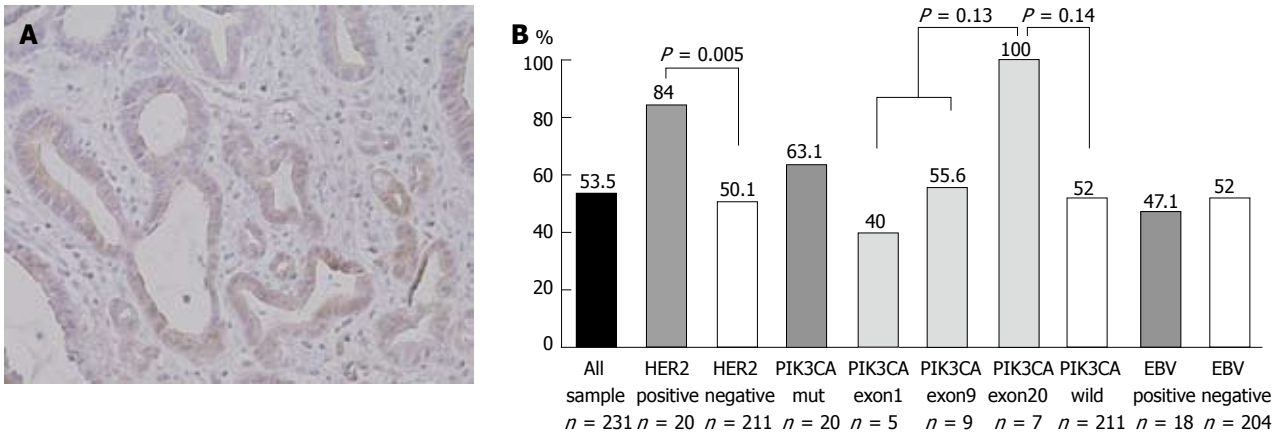

Figure 4 Immunohistochemical analysis and assessment of phospho Akt positivity based on molecular alterations in gastric cancer tissues. A: Gastric adenocarcinoma showing phospho Akt (pAkt) positivity. Original magnification, ×200; B: pAkt expression significantly correlates with human epidermal growth factor receptor 2 (HER2) overexpression ($P < 0.01$) but not with phosphatidylinositol 3-kinase, catalytic, alpha polypeptide (PIK3CA) mutations ($P = 0.37$) or Epstein-Barr virus (EBV) infection ($P = 0.69$).

0.36-2.31, $P = 0.98$).

Association of HER2 overexpression, PIK3CA mutations and EBV infection

One of our cases showed both HER2 overexpression and EBV infection and 3 cases showed both PIK3CA mutations and EBV infection. However there were no cases showing both PIK3CA mutations and HER2 overexpression. Three of the 4 cases were positive also for pAkt expression. PIK3CA mutations were present in 3 EBV-positive cancers, including 2 cases of LELC (2/5, 40%). One EBV-positive cancer with a PIK3CA mutation (H1047R) was MSI-positive.

pAkt expression

pAkt expression was positive in 119 (53%) of our cases but this showed no correlation with clinicopathological characteristics (Figure 4A). On the other hand, pAkt expression was found to be significantly correlated with HER2 overexpression (16/19 *vs* 103/204, $P < 0.01$) but not with PIK3CA mutations (12/19 *vs* 107/204, $P = 0.37$) or EBV infection (8/17 *vs* 103/198, $P = 0.69$) (Table 2). The frequency of pAkt expression was higher in cancers with exon20 mutations (100%) than in those with exon1 (40%) or exon9 (56%) mutations of PIK3CA, although this difference did not reach statistical significance (Figure 4B). The five-year survival rates were 37% in patients with pAkt expression and 59% in those without pAkt expression (HR 1.75; 95%CI: 1.12-2.80, $P = 0.02$) (Figure 5). Hence, pAkt expression significantly correlates with a poor prognosis in gastric cancer.

DISCUSSION

In our present study, we systematically characterized HER2 expression, PIK3CA mutations and EBV infection, all of which are involved in the PI3K-Akt signaling pathway, in a large cohort of patients with gastric cancer ($n = 231$). We aimed to determine the prevalence of these characteristics with a high level of precision and to correlate them with clinicopathological and molecular features, such as MSI and pAkt expression.

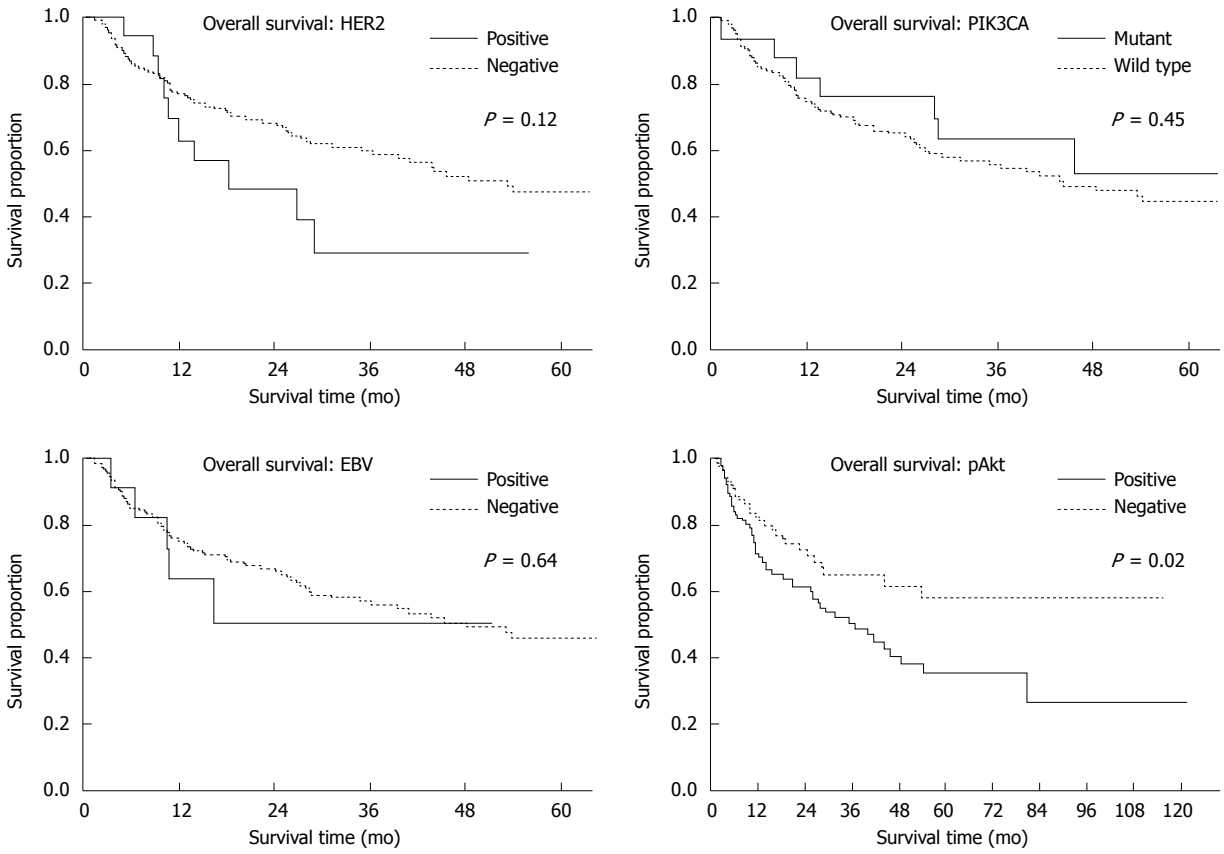

Figure 5 Survival analysis of gastric cancer patients. Three year survival of human epidermal growth factor receptor 2 (HER2)-positive *vs* HER2-negative, 29.1 mo *vs* 59.4 mo; Phosphatidylinositol 3-kinase, catalytic, alpha polypeptide (PIK3CA) mutation *vs* wild type, 63.7 mo *vs* 56.3 mo; Epstein-Barr virus (EBV)-positive *vs* EBV-negative, 51.3 mo *vs* 57.6 mo; And phospho Akt (pAkt)-positive *vs* pAkt-negative, 50.7 mo *vs* 64.8 mo. Five year survival of pAkt-positive *vs* pAkt-negative cases, 35.5 mo *vs* 58.1 mo.

HER2 overexpression (IHC 3+) was present in 20 samples (8.4%), a value that is within the range (7%-34%) reported in the current literature[5-9]. HER2 overexpression was found to significantly correlate with the intestinal histological type. Hence, the frequency of HER2 expression may depend on, at least in part, the distribution of histology in a cohort of gastric cancer samples. Some studies have suggested that HER2 positivity in gastric cancer is associated with poor outcomes and aggressive disease, but the results are conflicting. We found for the first time in our present analyses that HER2 overexpression significantly correlates with pAkt expression in gastric cancer tissues. Moreover, pAkt expression correlated with a poor prognosis in these patients. Thus, the HER2-Akt axis may play an important role in gastric cancer.

Pyrosequencing-based methods facilitate the identification of low-frequency tumor mutations and allow a more accurate assessment of tumor mutation burden[17,23,24]. We characterized PIK3CA mutations in gastric cancer tissues using pyrosequencing for the first time. The overall prevalence of PIK3CA mutations was found in our analysis to be 8.7%, a value that is within the previously reported range (4% to 25%)[10,12-15]. The mutation frequency was found to be high (21.4%) in T4 cancers and low (6.4%) in T2 cancers in our sample cohort. Thus, PIK3CA mutations appear to be late events in gastric carcinogenesis, leading to tumor progression. These patients might therefore be appropriate for targeted therapies directed against the PI3K pathway.

The most common PIK3CA mutation found in our analysis was H1047R, which was also found previously[15]. Importantly, two new types of mutations were found in exon1. To our knowledge, PIK3CA mutations involving residues 88 and 108 (R88Q and R108H) have been never reported previously in gastric cancer, nor described in the COSMIC database, despite the large number of previous studies in which this region was investigated. These mutations have been detected in several other types of cancer tissues[25]. Importantly also, these mutations have been reported to be gain-of-function[26-28]. Our present results thus have potential clinical implications since the mutational status of PIK3CA could stratify patients for genotype-based molecular therapies targeting the PI3K pathway. Hence, exon1 of PIK3CA should be analyzed in gastric cancer patients in these clinical settings.

PIK3CA mutations were found to be significantly associated with the MSI phenotype in our experiments. An association between PIK3CA mutations and MSI has been reported, or at least suggested, for both gastric and colon cancers[12,13,29]. We found in our present study that PIK3CA mutations in cancers with MSI are distributed in exon1, exon9 and exon20. These results further sup-

port the notion that PIK3CA is one of the most important oncogenes activated by missense mutations in MSI-positive gastric cancers.

The frequency of pAkt expression was found to be higher in cancers with exon20 mutations (100%) than in those with exon1 (40%) or exon9 (56%) mutations in PIK3CA. These results further support the notion that the functional significance of PIK3CA mutations depends on the mutation type and that the H1047R hotspot mutation has high oncogenic activity.

The previous ToGA study has shown that the addition of trastuzumab to the chemotherapeutic regimen improves survival in patients with advanced gastric or gastroesophageal junction cancer[5,6]. PIK3CA mutation is one of the mechanisms underlying the resistance to trastuzumab in breast cancer[30]. Trastuzumab is likely to be effective for HER2-overexpressing breast cancers with no PIK3CA mutations, with possible rescue using HER2-TKIs in cases of relapse[31]. For HER2-overexpressing breast cancer with PIK3CA mutations, inhibitors against molecules of the PI3K pathway are possibly more effective than anti-HER2 agents, which are unlikely to be beneficial[32]. In our present study, PIK3CA mutations were not found in gastric cancers with HER2 overexpression. Thus, it is unlikely that PIK3CA mutation is a major mechanism underlying the resistance to trastuzumab in gastric cancer.

HER2 overexpression was found in only one of the 18 EBV-positive gastric cancers in our sample cohort. This result can be explained, at least in part, by the fact that HER2 overexpression and EBV infection significantly correlate with intestinal and diffuse histological types, respectively. On the other hand, PIK3CA mutations were identified in 3 EBV-positive cancers, including 2 cases of LELC (2/5, 40%). Although not analyzed in our current study, EBV infection reportedly inactivates PTEN through the CpG island methylation of its promoter in EBV-associated gastric cancer[21]. Thus, alterations in the PI3K-Akt signaling pathway in EBV-positive gastric cancers may differ from those in EBV-negative cancers.

Finally, pAkt expression was found to correlate with a poor prognosis in gastric cancer. A significant association between increased pAkt expression and poor prognosis has been reported previously in patients with T3/T4 gastric cancer but not in those with T1/T2 cancer[33]. It has been reported also that pAkt expression is associated with increased resistance to multiple chemotherapeutic agents in gastric cancer patients, when chemotherapeutic sensitivities were tested using MTT assays[34]. Thus, Akt activation appears to lead to a poor prognosis and resistance to chemotherapeutic agents in gastric cancer. A positive correlation between a decrease in the pAkt levels after gefitinib administration and tumor apoptotic index in gastric cancer has also been reported[35]. Further analyses regarding the pAkt status in cancer tissues before and after chemotherapy and molecular targeted therapy will be necessary. Not all Akt activation events can be explained by HER2 expression, PIK3CA mutations, and EBV infection in gastric cancer. We have reported previously that a dominant negative insulin-like growth factor (IGF)-1 receptor blocks the Akt-1 activation induced by IGF-1 and IGF-2 in gastric cancer cell lines[36]. Thus, molecular alterations, such as the overexpression of IGF-1 receptor, might be involved in the activation of Akt in gastric cancer and this issue needs to be clarified in the near future.

COMMENTS

Background

Personalized therapy has begun also in advanced gastric cancer through the use of trastuzumab, an anti-human epidermal growth factor receptor 2 (HER2) antibody. Many drugs targeting the phosphatidylinositol 3-kinase (PI3K)-Akt pathway have now been developed and clinical trials are ongoing. An appropriate biomarker is necessary for successful molecular targeted therapy. The alterations of molecules in the PI3K-Akt pathway could be a good biomarker for such drugs.

Research frontiers

Various alterations, such as activation of growth factor receptors, PI3K, catalytic, alpha polypeptide (PIK3CA) mutations and Epstein-Barr virus (EBV) infection lead to activation of the PI3K-Akt signaling pathway. However, clinicopathological and molecular correlates among such alterations have not been clearly addressed. In the present study, the authors identify new clinicopathological and molecular correlations between HER2 expression, PIK3CA mutations, EBV infection and phospho Akt (pAkt) expression in gastric cancer.

Innovations and breakthroughs

This is the first study to systematically characterize HER2 expression, PIK3CA mutations and EBV infection, all of which are involved in the PI3K-Akt signaling pathway, in a large cohort of patients with gastric cancer. The prevalence of these characteristics was thereby determined with a high level of precision and correlations with the clinicopathological and molecular features of gastric cancers, such as microsatellite instability and pAkt expression, could be assessed accurately for the first time.

Applications

The results have potentially important clinical implications since the mutational status of PIK3CA can be used to stratify cancer patients for genotype-based molecular therapies that target the HERs-PI3K pathway.

Terminology

PI3K-Akt pathway: Akt is believed to transduce the major downstream PI3K signals in cancer. Akt regulates cell growth and survival pathways by phosphorylating substrates such as GSK3, forkhead transcription factors, and the TSC2 tumor suppressor protein; PIK3CA: PIK3CA encodes a key enzymatic subunit of PI3K. Gain of function mutations in PIK3CA occur frequently in several cancer types. Hotspots of PIK3CA mutations are located in exons 9 and 20.

Peer review

The authors investigated HER2 expression, PIK3CA mutations and EBV infection in patients with gastric cancer. The results demonstrated that pAkt expression significantly correlates with the prognosis and the HER2 expression status in gastric cancer. This article is important for the further development of molecular targeted therapy in patients with advanced gastric cancer.

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S- Editor Gou SX **L- Editor** A **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v18.i45.6587

World J Gastroenterol 2012 December 7; 18(45): 6587-6596
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Tumour seeding after percutaneous cryoablation for hepatocellular carcinoma

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Supported by Grants from the Key Scientific and Technological Research Foundation of the National Special-Purpose Program, No. 2008ZX10002-018; Military Special-Purpose Program, No. BWS11J074; and the Capital Medical Research and Development Fund, No. 2009-2041, China

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Received: June 10, 2012 Revised: July 30, 2012
Accepted: August 14, 2012
Published online: December 7, 2012

Abstract

AIM: To assess the rate and risk factors for tumour seeding in a large cohort of patients.

METHODS: Over an 8-year period, 1436 hepatocellular carcinoma (HCC) patients with 2423 tumour nodules underwent 3015 image-guided percutaneous cryoablation sessions [1215 guided by ultrasonography and 221 by spiral computed tomography (CT)]. Follow-up CT or magnetic resonance imaging was performed every 3 mo. The detailed clinical data were recorded to analyse the risk factors for seeding.

RESULTS: The median follow-up time was 18 (range 1-90) mo. Seeding was detected in 11 patients (0.76%) at 1-24 (median 6.0) mo after cryoablation. Seeding occurred along the needle tract in 10 patients and at a distant location in 1 patient. Seeded tumours usually showed similar imaging and histopathological features to the primary HCCs. Univariate analyses identified subcapsular tumour location and direct subcapsular needle insertion as risk factors for seeding. Multivariate analysis showed that only direct subcapsular needle insertion was an independent risk factor for seeding (P = 0.017; odds ratio 2.57; 95%CI: 1.47-3.65). Seeding after cryoablation occurred earlier in patients with poorly differentiated HCC than those with well or moderately differentiated HCC [1.33 ± 0.577 mo *vs* 11.12 ± 6.896 mo; P = 0.042; 95%CI: (-19.115)-(-0.468)].

CONCLUSION: The risk of seeding after cryoablation for HCC is small. Direct puncture of subcapsular tumours should be avoided to minimise seeding.

Key words: Cryoablation; Hepatocellular carcinoma; Tumour seeding; Clinical feature; Risk factor

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INTRODUCTION

In patients with hepatocellular carcinoma (HCC) superimposed on cirrhosis, orthotopic liver transplantation, surgical resection and percutaneous ablation are considered radical treatments as they provide better survival rates compared with no treatment[1]. Because of the poor acceptance of surgery and a severe shortage of donor organs, image-guided percutaneous ablation therapies play an important role in the management of HCC. Various local ablation therapies such as percutaneous ethanol injection (PEI), radiofrequency ablation (RFA), microwave (MW) ablation and cryoablation have been developed for the treatment of unresectable HCC. All these procedures require the insertion of long, sharp needles into the liver parenchyma and tumours, which may cause various complications, although the complication rates are low. Tumour seeding is one of the most serious complications, especially in patients who are waiting for liver transplantation[2]. The reported incidence of seeding after other ablation procedures varies widely: 0.2%-1.4% following PEI[3,4], 0.005%-12.5% following RFA[5,6] and 0.75% following MW ablation[7].

Certain factors have been found to increase the likelihood of needle-tract seeding, including a superficial or subcapsular tumour[8], high number of needle insertions[9,10], large needle bore[9,11], end-cutting needle[8,9], absent or thin layer of normal liver parenchyma surrounding the needle tract[9,10], high-grade HCC (moderately or poorly differentiated[3,8,9], high serum alpha-fetoprotein (AFP) level[2], tumour volumes > 2 cm^3 and immunosuppression[12].

Argon-helium cryoablation is a new local ablation modality. At one time, this technology caused some authors to question its use in HCC. Most of the bias against this percutaneous setting is based on a theoretical risk of post-procedure haemorrhage. However, the gradual downsizing of cryoprobes has fueled interest in percutaneous use, which offers several potential advantages versus the heat-based ablation modalities[13]. First, multiple cryoprobes can be used simultaneously to generate a large zone of ablation. Second, the size and shape of the developing ice ball can be readily visualized using intra-procedural computed tomography (CT), magnetic resonance imaging (MRI) or ultrasound (US). Third, in contrast to heat-based ablation, percutaneous cryoablation is a relatively painless procedure. Recently, many studies have reported that imaging-guided percutaneous cryoablation is safe and effective for the treatment of HCC[14-16]. In our previous study, the 1-, 2- and 3-year survival rates in patients with HCC < 5 cm in diameter who were treated with cryoablation were 92%, 82% and 64%, respectively, and the rate of serious complications was low[15]. To our knowledge, tumour seeding after percutaneous cryoablation for HCC has not been described to date. The present study was conducted to evaluate the incidence and possible risk factors for seeding after percutaneous cryoablation, by reviewing the prospective database of HCC patients treated by cryoablation in our department.

MATERIALS AND METHODS

Patients

This study included 1436 consecutive patients with HCC who were treated at the Center of Therapeutic Research for Hepatocellular Carcinoma, 302 Hospital of PLA, between April 2003 and June 2011. HCC was diagnosed based on typical findings on MRI or CT (hyperattenuation in the arterial phase and hypoattenuation in the portal-venous phase) and serum AFP level. The diagnosis was confirmed by histopathological examination of US- or CT-guided biopsy specimens in 736 patients. Until 2007, we biopsied almost all tumours before treatment, and after 2007 we only biopsied cases in which we could not make a definite diagnosis using dynamic CT or MRI. Biopsy specimens for histological examination were obtained with 1-2 passes of a 19.5-gauge end-cutting needle (Auto-Vac; Angiomed, Karlsruhe, Germany). Histopathological grading of tumour differentiation was performed using the criteria described by Edmondson *et al*[17]. Tumour stage was defined according to the Barcelona Clinic Liver Cancer (BCLC) classification[18]. Performance status (PS) was defined according to the Eastern Cooperative Oncology Group criteria (ECOG). The 1436 patients had a total of 2423 tumours with a diameter of 1.2-15.0 cm (mean 4.5 ± 2.3 cm). The clinical characteristics of the patients are shown in Table 1. All cryoablation treatments were approved by the Research Ethics Committee at 302 Hospital of PLA. Written informed consent was obtained from all patients who met the inclusion criteria, before blood and tumour specimens were obtained, and before data were collected and analysed.

Inclusion and exclusion criteria

Inclusion criteria for cryoablation were as follows: contraindications to surgical resection or orthotopic liver transplantation, Child-Pugh class A or B liver function, total serum bilirubin level < 51.3 μmol/L, platelet count ⩾ 20 × 10^9/L and performance status ⩽ 2. Ascites was controlled before the procedure with diuretics. Patients with early HCC who were reluctant to undergo hepatic resection or transplantation were included. Patients were excluded for the following reasons: BCLC stage D (ECOG PS > 2, Child-Pugh C), tumour thrombosis at the main branch of the portal vein and the size of the thrombosis exceeded 50% of the diameter of the portal vein, extrahepatic metastasis, tumours which were not accessible percutaneously, or a history of other ablation therapies. We generally performed percutaneous cryoablation in patients with up to three lesions, all of which were ⩽ 5 cm in diameter, but we performed a combination of repeated cryoablation and transarterial chemoembolisation (TACE) in some patients who did not meet these criteria.

Technical terms

We defined a session as a single treatment consisting of one or more ablations performed on one or more tumours. To assess tumour depth, we categorised tumours

Table 1 Baseline characteristics of patients *n* (%)

Baseline characteristics	Value
Age (yr)[1]	55.9 ± 9.2
Sex	
Male	1176 (81.9)
Female	260 (18.1)
Aetiology	
HBs-Ag positive only	1229 (85.6)
HCV-Ab positive only	168 (11.7)
Both positive	19 (1.3)
Both negative	20 (1.4)
Child-Pugh score	
Class A	874 (60.9)
Class B	562 (39.1)
Tumour size (cm)	
⩽ 3	411 (28.6)
3-5	656 (45.7)
⩾ 5	369 (25.7)
Tumour number	
Single	1213 (84.5)
2-3	223 (15.5)
Tumour location	
Subcapsular	484 (33.7)
No subcapsular	952 (66.3)
Route of needle insertion	
Direct subcapsular insertion	213 (14.8)
Deep	1223 (85.2)
BCLC staging	
Stage A	787 (54.8)
Stage B	453 (31.5)
Stage C	196 (13.7)
Completed ablation	
Yes	1168 (81.3)
No	268 (18.7)
Tumour differentiation[2]	
Well or moderate	490 (66.6)
Poorly	246 (33.4)
Biopsy performed prior to cryoablation[2]	736 (51.3)
Number of sessions	
Single	336 (23.4)
2	743 (51.7)
> 2	357 (24.9)
AFP (ng/mL)[1]	575 ± 2039

[1]Values are expressed as mean ± SD, n = 1436; [2]Of 736 cases in which biopsy was performed. HBs-Ag: Hepatitis B surface antigen; HCV-Ab: Hepatitis C virus antibody; BCLC: The Barcelona Clinic Liver Cancer classification; AFP: Alpha-fetoprotein.

as subcapsular or deep. Tumours were defined as subcapsular when they were located adjacent to the surface of the liver, less than 0.5 cm of parenchyma between the tumour and the liver capsule, otherwise, they were defined as deep. Direct subcapsular needle insertion was defined as puncture of subcapsular tumours without traversing a sufficient portion of normal hepatic parenchyma. The number of needle insertions was defined as the total number of needle positions in all sessions.

Argon-helium cryoablation procedure

Argon-helium cryoablation was performed as described in our previous report[16]. Briefly, an argon-helium gas-based CRYOcare system (EndoCare, Irvine, CA, United States) and cryoprobes were used to freeze the tumour with a dual freeze-thaw cycle under US or CT guidance. After determining the most favourable percutaneous approach, we inserted the 3-mm cryoprobe into the tumour through the sheath introducer system under US or CT guidance, and advanced the tip to the distal margin of the targeted lesion. The number of probes used depended on the location and size of the lesions to be ablated. The dual freeze-thaw cycle comprised a 20-min freeze, a 10-min thaw and a further 15-min freeze. The dimensions of the frozen tissue were monitored by US or CT. The cryoprobe temperatures were reduced to -135 ℃ ± 2 ℃ within 1 min. After removal of the probes, all tracts were packed with Surgicel (Johnson and Johnson, Inc., Arlington, TX, United States) through the sheath introducer to control bleeding, and the sheath introducer was removed. We aimed to perform curative ablation of all tumours in each session by single or multiple cryoablation, particularly for tumours < 5 cm in diameter. Dynamic CT or MRI was performed 2-3 d after treatment to evaluate treatment efficacy. Complete ablation was defined as non-enhancement of the entire lesion on CT or MRI with a safety margin in the surrounding liver parenchyma. Patients underwent additional ablation sessions until complete ablation was confirmed in all nodules, to a maximum of three sessions. If ablation was incomplete after three sessions, we performed TACE. The cryoablation procedure was performed under conscious sedation. Echocardiography, ventilation and oxygen saturation levels were monitored throughout the procedure. Patients were kept warm during cryoablation with warming mats.

Follow-up and tumour seeding

All patients underwent MRI and CT at 1 mo after cryoablation. Patients were then assessed every 2-3 mo, including measurements of liver biochemistry and AFP level, and by CT or MRI. A newly detected tumour attached to the peritoneum or pleura was considered to be seeded, and the diagnosis was confirmed by biopsy and histopathological examination. Seeded tumours were treated with repeat cryoablation, PEI or TACE when feasible. The seeding rate was calculated based on the number of patients.

Statistical analysis

Potential risk factors for seeding were analysed. The following variables were recorded: age, sex, viral markers, tumour size, number of tumour nodules, tumour location, direct subcapsular needle insertion, tumour differentiation, number of cryoablation sessions, number of needle insertions, percutaneous biopsy prior to cryoablation and serum AFP level. Continuous variables were compared between patients with and without seeding using the Student's t test. The χ^2 test or Fisher's exact test was used to compare categorical variables between the groups. Variables with $P < 0.1$ were entered into a multivariate logistic regression model using stepwise selection of variables. Variables with $P < 0.05$ were considered statistically significant. All analysis were conducted using SPSS software version 13 (SPSS Inc., Chicago, IL, United States).

Table 2 Characteristics of the 11 hepatocellular carcinoma patients who had tumour seeding after cryoablation

Case No.	1	2	3	4	5	6	7	8	9	10	11
Age (yr)	66	51	47	51	65	43	49	61	58	58	72
Sex	M	F	M	M	F	M	M	M	M	F	M
Child-Pugh class	A	A	A	A	B	A	A	B	A	A	B
BCLC Stage	A	B	B	B	B	C	A	B	A	A	B
AFP (ng/mL)	7	9	3550	368	8589	16	75	33	23	48	294
No. of tumours	1	1	1	1	1	1	1	3	1	2	1
Tumour size (cm)	6	3	5.4	6	3.2	8	2.4	4.8	2.6	2	5.6
No. of sessions	2	1	4	3	1	3	1	2	1	4	2
No. of needle insertions	4	2	6	4	2	6	1	3	1	4	3
Completed ablation	Yes	Yes	No	No	Yes	No	Yes	Yes	Yes	Yes	Yes
Biopsy	No	Yes	Yes	Yes	Yes	No	No	No	No	Yes	Yes
Tumour differentiation											
Primary HCC	-	Poor	Mod	Mod	Poor	-	-	-	-	Well	Poor
Seeding HCC	Mod	Poor	Mod	Mod	-	Mod	Mod	Mod	Mod	Well	-
Subcapsular location	Yes	No	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	No
Direct subcapsular insertion	Yes	No	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	No
Seeding location	Ip	Ip	Sf	Ip	Pleura	Ip and Im	Ip	Ip	Ip	Ip	Pc
Seeding time (mo)	24	2	5	5	1	6	12	7	12	18	1
Overall survival (mo)	36	25	9	19	18	12	18	26	36	60	5
Prognosis	Died	Alive	Died	Died	Died	Alive	Alive	Died	Alive	Alive	Alive

M: Male; F: Female; Ip: Intraperitoneal; Sf: Subcutaneous fat; Im: Intercostal muscle; Pc: Peritoneal cavity; HCC: Hepatocellular carcinoma; BCLC: The Barcelona Clinic Liver Cancer classification; AFP: Alpha-fetoprotein; Mod: Moderate.

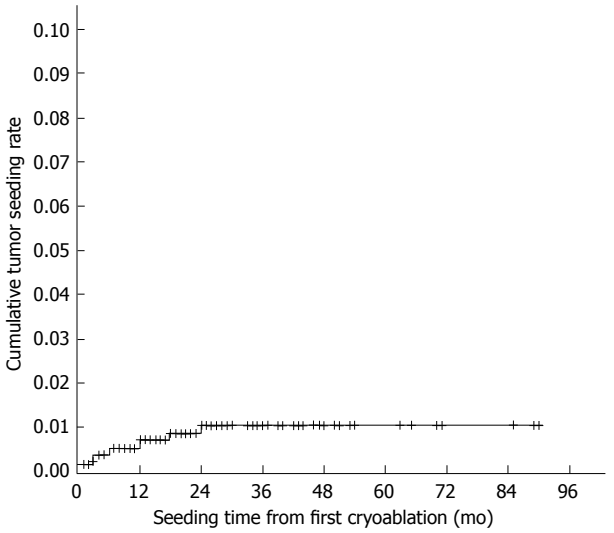

Figure 1 Cumulative tumour seeding rate. The cumulative rate was 0.49% at 1 year and 1.0% at 2 years.

RESULTS

Clinical features of patients with tumour seeding

A total of 1436 HCC patients underwent 3015 cryoablation sessions (1215 guided by ultrasonography and 221 guided by CT; average 2.1 sessions per patient) for 2423 nodules. When a patient underwent more than one treatment session, the data from the initial session were used. During the follow-up period (median 18 mo; range 1-90 mo), seeding was diagnosed in 11 patients at an interval of 1-24 (median 6.0) mo after the first cryoablation. The seeding rate was 0.76% per patient (11/1436). The longest interval between the first cryoablation session and detection of seeding was 2 years. The cumulative seeding rates were analysed using Kaplan-Meier estimates, and were 0.49% at 1 year and 1.0% at 2 years (Figure 1). Table 2 shows the baseline characteristics of the 11 patients with seeding. Eight of these patients were male, and the mean patient age was 56.5 ± 9.0 years. Ten patients were hepatitis B surface antigen positive and one patient was hepatitis C virus antibody positive. Eight patients were Child-Pugh class A and three were Child-Pugh class B. The mean tumour size was 4.5 ± 1.9 cm, and the mean number of tumours was 1.3 ± 0.6. The mean number of needle insertions was 3.3 ± 1.7. Direct subcapsular needle insertions were performed in eight patients with subcapsular tumours. Six patients underwent biopsy prior to cryoablation, of which three had poorly differentiated HCC. Four patients were classified as BCLC stage A, six as stage B, and one as stage C. The tumours were completely ablated in eight patients. The mean serum AFP level was 1182.9 ± 2668.6 ng/mL.

Seeding occurred along the cryoablation needle tract in 10 patients, and at a distant location in 1 patient (Figure 2). The seeding was intraperitoneal in seven patients, intraperitoneal and in the intercostal muscles in one patient, pleural in one patient, and in the abdominal wall (subcutaneous fat) in one patient. One patient had distant seeding in the peritoneal cavity. In ten patients, the seeded tumours were < 3 cm in diameter, and in one patient the tumour was 3 cm in diameter. Nine (81.8%) patients had a single seeded tumour, and the other two (18.2%) patients had two and three seeded tumours, respectively, indicating that multiple seeding was not uncommon. One patient developed treatment-related liver haemorrhage 5 mo before the seeding was detected.

CT and MRI are the preferred imaging modalities for detecting needle-tract seeding. The seeded tumours are usually detected as one or a few round or oval-shaped

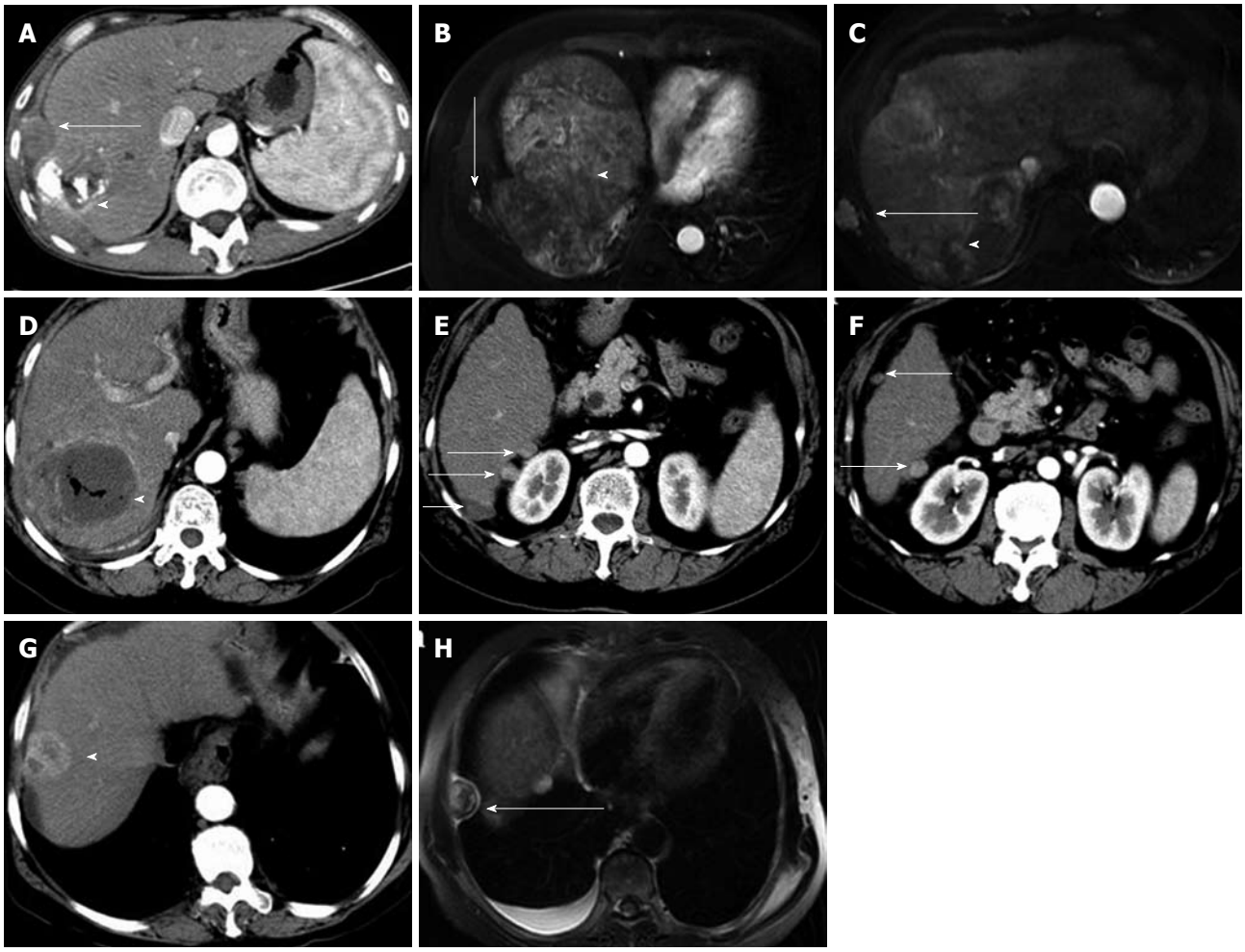

Figure 2 Locations of hepatocellular carcinoma and of seeding after cryoablation. A: Intraperitoneal seeding (case 4); B: Intraperitoneal and intercostal muscle seeding (case 6); C: Seeding in subcutaneous fat (case 3); D: Complete ablation of primary hepatocellular carcinoma (HCC) (case 11); E and F (case 11): Multiple, small, intraperitoneal seeding nodules 1 mo later; G: Complete ablation of primary HCC (case 5); H: Pleural seeding nodules 1 mo later (case 5). Arrowheads indicate primary HCCs, arrows indicate seeding.

enhancing nodules along the needle tract, with a few occurring at a distant location. Seeded tumours showed a similar imaging pattern to primary HCCs, with arterial phase hyperattenuation followed by portal-venous phase hypoattenuation (Figure 3).

Tumour biopsies performed before cryoablation in six patients who developed seeding showed that one patient had a well differentiated tumour, two patients had moderately differentiated tumours, and three patients had poorly differentiated tumours. The seeded tumours in the nine patients without distant or pleural seeding were confirmed by biopsy and histopathological examination. The seeded tumour showed similar differentiation features to the primary HCC in four of these patients (Figure 4).

Seeding was treated by PEI in four patients, resection in one patient, cryoablation in two patients, cryoablation plus sorafenib in two patients, and conservative treatment in two patients. Of the nine patients with seeding who were treated, recurrence of seeding after treatment occurred in five (55.6%), including three treated with PEI and two treated with cryoablation plus sorafenib. Three of the patients with recurrent seeding were treated with RFA plus radiation and did not have further recurrence, and the other two patients were treated with sorafenib plus cryoablation and TACE (Figure 5).

At the end of the follow-up period, five patients with seeding had died. The causes of death were intrahepatic HCC progression and liver failure. No patient died of their seeded tumour nodules. In patients with seeding, the cumulative survival rates were 90% at 1 year, 68% at 2 years, 53% at 3 years, 32% at 4 years and 32% at 5 years. In patients without seeding, the cumulative survival rates were 86% at 1 year, 61% at 2 years, 51% at 3 years, 43% at 4 years and 34% at 5 years. There were no significant differences in survival rates between the two patient groups (P = 0.942) (Figure 6).

Risk factors for tumour seeding

Table 3 shows the results of the univariate analysis to identify risk factors for seeding. Direct subcapsular needle insertion (P = 0.0043) and subcapsular tumour location (P = 0.0152) were associated with seeding. There were no significant associations between seeding and age, sex,

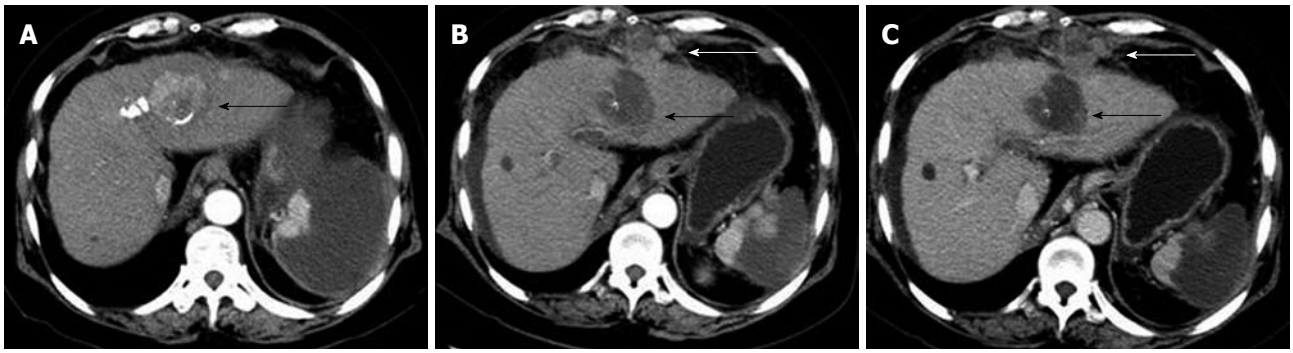

Figure 3 Computed tomography showing tumour seeding in case 2 after cryoablation for hepatocellular carcinoma, with a history of transcatheter arterial chemoembolisation. A: Contrast-enhanced computed tomography (CT) image showing a 3-cm diameter hepatocellular carcinoma (HCC) in segment Ⅱ before cryoablation (black arrow); B and C: Contrast-enhanced CT images during the arterial phase (B) and portal-venous phase (C) showing intraperitoneal seeding (white arrow) at 2 mo after biopsy and percutaneous cryoablation. The tumour showed hyperattenuation during the arterial phase and hypoattenuation during the portal-venous phase, similar to the primary HCC. Histopathological examination of the seeded tumour showed a poorly differentiated HCC. Note that the intrahepatic tumour was completely ablated.

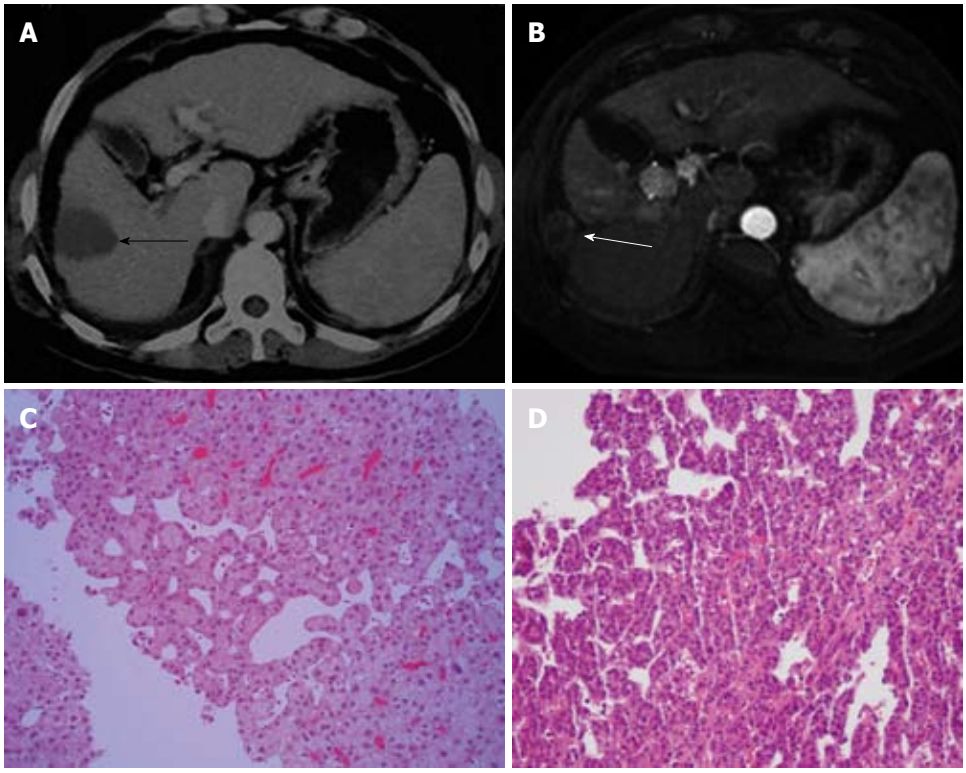

Figure 4 Computed tomography image and histopathology of primary and seeded tumours (case 10). The 58-year-old woman underwent biopsy and percutaneous cryoablation for a 2-cm diameter subcapsular hepatocellular carcinoma (HCC). A: The tumour was completely ablated (black arrow); B: Intraperitoneal seeding at 18 mo after treatment (white arrow); C: Histopathological examination of the biopsy specimen from the primary tumour showed a well differentiated HCC; D: The seeded tumour was resected, and histopathological examination showed a well differentiated HCC. The patient was alive and tumour-free at 60 mo after cryoablation.

viral markers, Child-Pugh class, tumour size, number of nodules, number of sessions, number of needle insertions, tumour differentiation, biopsy prior to cryoablation, BCLC stage, incomplete ablation, or serum AFP level. Even though there was no significant association between seeding and tumour differentiation, seeding was detected earlier in patients with poorly differentiated HCC than in those with well or moderately differentiated HCC [1.33 ± 0.577 mo *vs* 11.12 ± 6.896 mo; 95%CI: (-19.115)-(-0.468); $P = 0.042$)]. Multivariate analysis showed that the only significant risk factor for seeding was direct subcapsular needle insertion (odds ratio 2.57; 95%CI: 1.47-7.65; $P = 0.017$).

DISCUSSION

The new modality of imaging-guided percutaneous argon-helium cryoablation has been widely developed in China. Many studies have reported the safety and efficacy of this technique in the treatment of HCC[14-16]. Although many complications have been reported, the majority are minor and can be treated conservatively. In carefully selected patients, the rate of serious complications is low[16]. Because of its minimal invasiveness and resulting large ablation zone, percutaneous cryoablation is a useful treatment modality for HCC[19].

However, occasional tumour seeding after percuta-

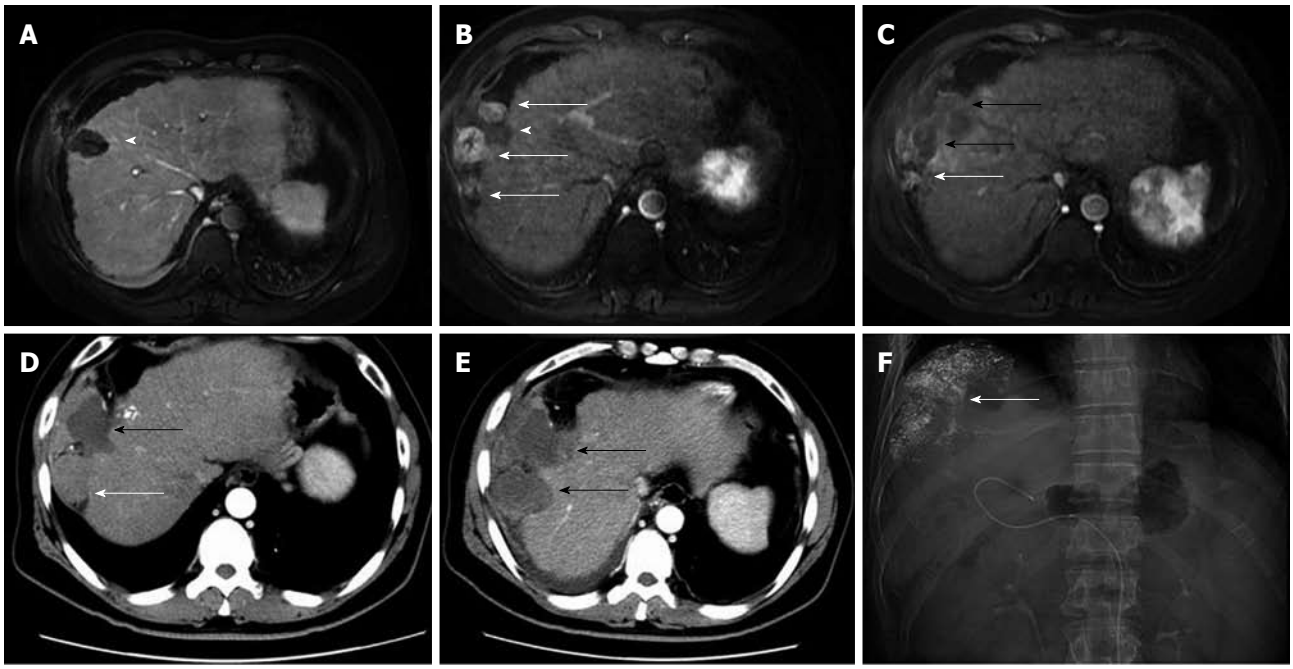

Figure 5 Patient treated with sorafenib plus cryoablation and transcatheter arterial chemoembolisation for hepatocellular carcinoma seeding (case 7). A: Complete ablation of the subcapsular hepatocellular carcinoma (arrowhead); B: Multiple, small, intraperitoneal seeded nodules (white arrows); C: Seeded nodules were treated with cryoablation (black arrows) plus sorafenib; D: Recurrence of seeding (white arrow); E: The recurrent seeding was treated with cryoablation (black arrows); F: The seeding was also treated with transarterial chemoembolisation (white arrow). Six months later, there was no further recurrence.

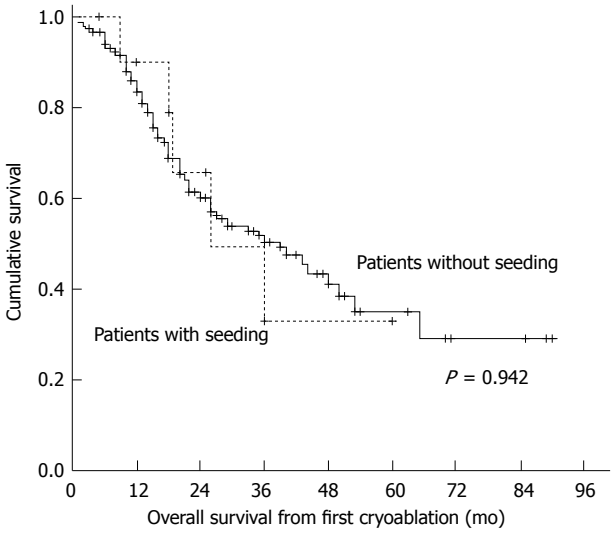

Figure 6 Cumulative overall survival in patients with and without tumour seeding. Survival curves are drawn according to the Kaplan-Meier method, using the log-rank test.

neous cryoablation for HCC remains unavoidable, as in other local ablation treatments such as PEI, RFA and MW ablation. In this study, we systematically searched for evidence of seeding using state-of-the-art imaging over a long follow-up period, and found seeding in 11 of 1436 patients treated with 3015 cryoablation sessions. The seeding rate was 0.76% per patient. The median interval between the first cryoablation session and detection of seeding was 6 (range 1-24) mo, with a median follow-up time of 18 (range 1-90) mo. At the end of the follow-up period, 580 patients had died, and these patients had a median survival time of 15 (range 1 to 65) mo. All patients were under close observation, and no patients were lost to follow-up. Because of the duration and quality of follow-up, the likelihood of having missed a seeded tumour in this study is minimal.

Similar to other percutaneous interventions such as biopsy, PEI and RFA, the sites of seeding after cryoablation were the thoracic wall, abdominal wall, diaphragm and peritoneal cavity[20-22]. Seeding usually occurred along the needle tract, but a few cases were at a distant location, with pleural and peritoneal cavity seeding in one patient each. Regular follow-up with contrast-enhanced CT or MRI from the chest to the pelvis is therefore very important.

The median time to diagnosis of seeding has been reported to be 13 (range 1-58) mo after biopsy[21], 6 (range 2-48) mo after PEI[21] and 28.5 (range 8.6-60.7) mo after RFA[22]. In the present study, the median time to diagnosis was 6.0 (range 1-24) mo after cryoablation. The longest interval from the first cryoablation session to the diagnosis of seeding was 2 years. It can therefore be concluded that it is necessary to carefully monitor patients for at least 2 years after cryoablation for HCC. The reason for the longest interval to the diagnosis of seeding in the present study being shorter than in previous studies on biopsy, PEI and RFA is unknown. There are no reports of growth rates for seeded tumours after cryoablation, but it has been reported that the growth rate of needle-tract seeding after biopsy varies depending on the initial number of implanted tumour cells and the doubling time of the tumour, as well as the microenvironment surrounding the seeded tumour. The doubling time of seeded tumours after bi-

Table 3 Characteristics of patients with and without tumour seeding

Variable	Seeding was identified (n = 11)	Seeding was not identified (n = 1425)	P value
Age (yr)[1]	56.5 ± 9.0	55.5 ± 9.3	0.8970
Gender (male/female)	8/3	1168/257	0.6895
HBs-Ag positive only	10	1219	0.9412
HCV-Ab positive only	1	167	1.0000
Both positive	0	19	1.0000
Both negative	0	20	1.0000
Child-Pugh class (A/B)	8/3	866/559	0.6176
Tumour size (cm)[1]	4.5 ± 1.9	4.6 ± 3.0	0.8800
Number of tumours[1]	1.3 ± 0.6	1.3 ± 0.7	0.9520
Number of sessions[1]	2.2 ± 1.2	2.3 ± 1.1	0.8764
Number of needle insertions[1]	3.3 ± 1.7	3.8 ± 1.1	0.3430
Direct subcapsular insertion	8	205	0.0043
Subcapsular location	8	476	0.0152
Biopsy performed[2]	6	730	0.8264
Poorly differentiated tumour	3	243	0.6674
BCLC stage (A/B/C)	4/6/1	783/447/195	0.2869
Completed ablation (yes/no)	8/3	1161/264	0.7235
AFP (ng/mL)[1]	1182.9 ± 2668.6	577.0 ± 2038.1	0.3270

[1]Values are expressed as mean ± SD; [2]Of 736 cases in which biopsy was performed. HBs-Ag: Hepatitis B surface antigen; HCV-Ab: Hepatitis C virus antibody; BCLC: The Barcelona Clinic Liver Cancer classification; AFP: Alpha-fetoprotein.

opsy is 112 (range 22-415) d[8], which is comparable to that of primary HCC. Regarding differentiation features, Matsukuma *et al*[23] reported that peritoneal seeding can infrequently proliferate aggressively with more differentiated features. In the current study, four patients with seeding showed similar tumour differentiation features to the primary HCC, and only three patients had poorly differentiated HCC, so it is difficult to explain the shorter interval in terms of tumour differentiation. The available data report a median HCC diameter of 32.5 mm in patients who underwent PEI and 30 mm in patients who underwent RFA, and the use of 14- to 22-gauge needles for biopsies, 21- to 22-gauge needles for PEI and 14- to 17-gauge needles for RFA[20,21]. The shorter interval to detection of seeding after cryoablation may be related to the use of larger needles (a 3-mm cryoprobe correlates to an 11-gauge needle) and larger nodule size (mean 45 ± 19 mm).

HCC is particularly prone to seeding, with higher seeding rates after biopsy (0%-5.1%)[24] than other solid tumours such as pancreatic tumours (0.003%-0.017%) and other abdominal tumours (0%-0.03%)[9,25].

In a recent review, several factors were suggested to contribute to seeding after percutaneous interventional procedures, which were listed as follows: poorly differentiated tumour, high serum AFP level, subcapsular tumour location, biopsy prior to RFA, high number of sessions and high number of electrode placements[19]. However, only Shirai *et al*[22] and Imamura *et al*[26] have reported multivariate analyses of these factors. Imamura *et al*[26] reported that only poor tumour differentiation was an independent risk factor for seeding. Shirai *et al*[22] reported that only RFA was an independent risk factor. In the present study, univariate analyses identified subcapsular tumour location and direct subcapsular needle insertion as risk factors. There were no significant associations between seeding and age, sex, viral markers, Child-Pugh class, tumour size, number of nodules, number of sessions, number of needle insertions, tumour differentiation, biopsy prior to cryoablation, BCLC stage, incomplete ablation or serum AFP level. Multivariate analysis showed that only direct subcapsular needle insertion was an independent risk factor for seeding.

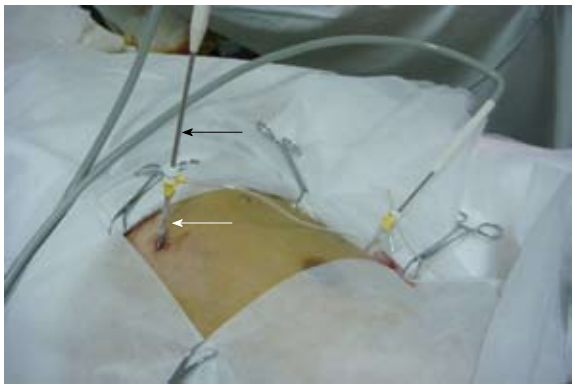

Figure 7 The sheath introducer system. The cryoablation needle (black arrow) is inserted and removed through the sheath introducer system (white arrow).

Several studies have reported that subcapsular tumour location was a risk factor for seeding[2,27-29]. In a study reporting a 12.5% seeding rate after RFA, all patients with seeding had a subcapsular tumour[2]. This is consistent with the results of our univariate analysis. In our initial experience, percutaneous cryoablation of subcapsular tumours was also associated with liver haemorrhage[16]. In the present study, treatment-related liver haemorrhage occurred in one patient with seeding. We therefore insert our cryoprobe across a portion of normal hepatic parenchyma, and avoid direct subcapsular needle insertion for subcapsular tumours whenever possible. This minimises both liver haemorrhage and needle-tract seeding. This may explain why multivariate analysis only identified direct subcapsular needle insertion as an independent risk factor.

There is still controversy regarding whether tumour biopsy prior to treatment or a poorly differentiated tumour increase the risk of seeding[21-23,30]. In this study, biopsy and a poorly differentiated tumour were not associated with a higher rate of seeding. The current study also did not show a significant association between seeding and tumour size or incomplete cryoablation, which is consistent with the findings of other studies[21,26]. Although the 3-mm cryoprobe was large, the risk of seeding after cryoablation was small. The risk of seeding may be reduced by the use of the sheath introducer system (Figure 7), through which cryoablation needles are inserted and removed. Similarly, Maturen *et al*[31] reported that no seeding occurred when they used a needle introducer that remained in position during multiple passes of a coaxial cutting needle for biopsies, which may protect the tissue along the needle tract and reduce seeding. Further studies should be conducted

to assess the effects of the sheath introducer system on the risk of seeding in percutaneous cryoablation for HCC. In addition, the low risk of cryoablation may be related to the mechanisms of cryoablation. Cryotherapy is believed to kill cells by several mechanisms, including intracellular ice formation, solute-solvent shifts that cause cell dehydration and rupture, small vessel obliteration causing hypoxia and specific anti-tumour immunoreactions that limit tumour growth[32,33]. Several studies found that cryoablation resulted in both local tumour necrosis and necrosis and shrinkage of the tissues adjacent to the tumour, which was thought to indicate ectopic tumour suppression[33]. Preclinical evidence of a cryo-immunologic response as well as some clinical data indicate that cryoablation may generate an anti-tumour response[34,35]. Our previous study indicated that cryoablation not only directly destroys malignant tissues, but also has effects on the adjacent tissues[36]. Cryotherapy resulted in reduced numbers of peripheral Treg cells and a lowering of the CD8-FoxP3+/CD8+FoxP3- ratio in malignant tissues[37]. We therefore speculate that anti-tumour immunoreactions induced by cryoablation may limit seeding. This concept deserves further study.

Although poorly differentiated tumour did not increase the risk of seeding, we found that seeding occurred earlier in patients with poorly differentiated HCC than in those with well or moderately differentiated HCC. It is possible that poorly differentiated HCC lacks cohesiveness[17] and grows more rapidly, allowing the seeding to be identified earlier.

It is not clear whether seeding affects prognosis. Shirai *et al*[22] and Imamura *et al*[26] investigated the prognosis of HCC patients with seeding after RFA. They reported that the survival rate was not particularly low in patients with seeding, and that seeding itself did not directly affect survival. The present study also did not find significant differences in the cumulative survival rates of patients with and without seeding. By the end of the follow-up period, five patients with seeding had died of intrahepatic HCC progression and liver failure. No patient died due to the growth of seeded nodules. Nevertheless, the survival rates of patients with seeding tended to be lower from the second year onwards. The reasons for the lack of significant differences in survival rates may be as follows: First, the number of patients with seeding was very small compared with the number without seeding. Second, seeded tumours were treated radically, which may improve outcome[7,38]. It is therefore impossible to claim that seeding does not affect prognosis, and seeded tumours should be treated with the aim of achieving local cure. It is essential to recognise that seeding is difficult to treat successfully. In the present study, the recurrence rate after local radical treatment of seeding was 55.6%.

In conclusion, the relatively low rate of tumour seeding after cryoablation for HCC is considered an acceptable clinical risk. Direct puncture of subcapsular tumours was found to be a risk factor for seeding. Although seeding is sometimes unavoidable, strict attention to detail and knowledge of seeding and its risk factors are helpful for minimising its occurrence.

COMMENTS

Background

Imaging-guided percutaneous argon-helium cryoablation is widely used in China, and this technique has been found to be safe and effective for the treatment of hepatocellular carcinoma (HCC). However, details of tumour seeding after this procedure have not been reported to date, even though seeding is one of the most important complications.

Research frontiers

This study reports the rate of tumour seeding after percutaneous cryoablation and analyses the risk factors for seeding in a large cohort of HCC patients who were treated with cryoablation sessions over an 8-year period.

Innovations and breakthroughs

Seeding occurred in 11 (0.76%) of 1436 patients treated with percutaneous cryoablation in this study. Only direct puncture of a subcapsular tumour was an independent risk factor for seeding.

Applications

This study indicates that the risk of seeding after percutaneous cryoablation for HCC is small and is considered an acceptable clinical risk. This procedure is minimally invasive and results in a large ablation zone, making it a useful treatment modality for HCC. However, direct puncture of a subcapsular tumour should be avoided. The small risk of seeding may be due to the use of an introducer sheath, or to the mechanisms of cryoablation, and further research is warranted.

Terminology

Percutaneous cryoablation requires the insertion of needle into the liver parenchyma and tumour, which may cause tumour seeding. However, the incidence of HCC seeding after the procedure is low.

Peer review

The authors analyzed the incidence of HCC tumour seeding after percutaneous cryoablation. It is very interesting study and has a great scientific value for physicians who take care of patients with this pathology. The study is well designed and data is convincing.

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S- Editor Shi ZF **L- Editor** Webster JR **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v18.i45.6597

World J Gastroenterol 2012 December 7; 18(45): 6597-6604
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Clinical significance of human kallikrein 12 gene expression in gastric cancer

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Supported by Scientific Research Fund from Shanghai Science and Technology Committee, No. 08411964200
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Received: June 24, 2012 Revised: September 25, 2012
Accepted: September 29, 2012
Published online: December 7, 2012

Abstract

AIM: To investigate whether the expression of kallikrein 12 (KLK12) is related to the development of gastric cancer (GC) and to determine the role of KLK12 in gastric cancer cells growth, invasion and migration.

METHODS: Between September 2007 and March 2008, 133 patients with histologically confirmed GC were recruited for the study. Expression of KLK12 was detected in samples from GC patients by quantitative real-time reverse transcription polymerase chain reaction and immunohistochemistry. The relationship between KLK12 protein expression and clinicopathological features of GC was analyzed. The difference in 5-year survival rates between the high KLK12 protein expression group and the low KLK12 expression group was compared. Additionally, the expression of KLK12 was examined in various human GC cell lines, including MKN-28, SGC-7901 and MKN-45. Small interfering RNA (siRNA) was used to inhibit KLK12 expression in MKN-45 cells. Cell clones stably transfected with KLK12 siRNA were tested for KLK12 expression by quantitative real-time reverse transcription-polymerase chain reaction and Western blotting. Furthermore, a series of functional assays were performed in this study to assess the biological features of transfected cells. Cell proliferation was assessed using the methylthiazolyltetrazoliumassay. Finally, cell migration and invasion were assessed using transwell chamber assays.

RESULTS: Of the 133 GC patients included in the study, 126 (94.7%) showed a higher expression level of KLK12 mRNA when compared to noncancerous tissue specimens. Expression of KLK12 mRNA was significantly higher in GC tissues than in normal tissue ($P < 0.001$). KLK12 protein expression was detected in 96 of 133 (72.2%) GC samples with moderate or strong staining primarily in the cytoplasm. In contrast, negative immunostaining for KLK12 protein was observed in the corresponding normal gastric mucosal tissue. Overexpression of KLK12 protein was significantly associated with lymph node metastasis ($P = 0.001$), histological type ($P < 0.001$) and tumor-node-metastasis stage ($P = 0.005$), while no significant correlation was observed between expression of KLK12 protein and sex, age, depth of invasion, tumor size or lymphatic invasion. Furthermore, patients with high KLK12 expression had a significantly poorer 5-year survival rate than those with low KLK12 expression ($P = 0.002$). Expression of KLK12 mRNA was significantly higher in MKN-45 GC cells compared to normal mucosal cells or two other GC cell lines ($P < 0.01$). Expression of KLK12 in MKN-45 cells was downregulated after transfection with siRNA. Knockdown of KLK12 markedly decreased the proliferation of MKN-45 cells when compared with parent or mock-transfected cells ($P = 0.001$), especially from the 3rd to the 5th day of the assay. In migration assays, fewer KLK12 siRNA cells migrated through the chambers (22.00 ± 1.81) when compared to the parent (46.47 ± 2.42) or mock-transfected cells (45.40 ± 1.99); these differences were statistically sig-

nificant ($P < 0.001$). However, in the invasion assay, the number of KLK12 siRNA cells that invaded the chambers was 18.40 ± 1.12, closely similar to both the parent (18.67 ± 0.98) and mock-transfected cells (18.53 ± 0.92). There was no significantly difference between the three groups in the invasion assay ($P = 0.054$).

CONCLUSION: The *KLK12* gene is markedly overexpressed in GC tissue, and its expression status may be a powerful prognostic indicator for patients with GC. KLK12 might serve as a novel diagnosis and prognosis biomarker in GC.

Key words: Gastric cancer; Human kallikrein 12; Immunohistochemistry; Prognosis; Small interfering RNA; Migration; Invasion

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Zhao EH, Shen ZY, Liu H, Jin X, Cao H. Clinical significance of human kallikrein 12 gene expression in gastric cancer. *World J Gastroenterol* 2012; 18(45): 6597-6604 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i45/6597.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i45.6597

INTRODUCTION

Gastric cancer (GC) is the fourth most common malignancy, and the second most common cause of cancer mortality worldwide[1]. Although morbidity and mortality rates for GC are steadily decreasing steadily in many countries, the overall outcomes for patients with GC have not changed significantly in recent decades[2]. Additionally, none of the potential biomarkers proposed throughout the years for GC have presented the desired properties to be incorporated into routine clinical practice. The human tissue kallikrein (*KLK*) genes are a newly identified subgroup of putative serine proteases, consisting of 15 genes located within approximately 256 kb on chromosome 19q13.3-4[3-5]. Due to their protease activity and expression in many tissues and cell types, KLKs have been implicated in a wide range of physiological processes and the pathogenesis of human diseases[6,7]. Over the last several decades, a steadily increasing number of studies have suggested that human KLKs are involved in human carcinogenesis and that several KLKs may be promising biomarkers of prostate, ovarian, testicular, and breast cancers[8-10]. The most notable kallikrein protein biomarker is KLK3, also known as prostate specific antigen (PSA)[11]. PSA is currently the only biochemical test for prostate cancer, although the specificity of the test is not optimal[12]. Recently, a considerable amount of work has focused on identifying novel KLK-derived molecular markers of GC[13-16]. The human *KLK12* gene is a member of the KLK family, encoding human kallikrein 12 protein (hK12). Similar to other kallikreins, KLK12 is an enzyme with serine protease activity that participates in several biological processes[17]. Moreover, some researchers have shown that KLK12 might also play a role in human carcinogenesis[17-19]. However, no information is available regarding KLK12 expression in human GC.

To explore the vital role of KLK12 in the tumorigenesis and progression of GC, we examined expression patterns of KLK12 in GC tissues, analyzed the relationship between hK12 expression and clinicopathological factors of GC. Furthermore, a series of function assays utilizing small interfering RNA (siRNA)-mediated downregulation of KLK12 expression were performed.

MATERIALS AND METHODS

Patients and samples

Prior to operation, no patient had received any type of treatment. All research examinations were approved by the Ethics Committee Board of Renji Hospital. Moreover, participants in this study signed an informed consent form so that their samples could be used for research purposes from September 2007 to March 2008. A computerized database with the medical history of each patient was created for an extensive statistical analysis. Selection criteria included confirmation of GC diagnosis by histopathology and the availability of sufficient tumor tissue for RNA extraction. Tumor stage was defined according to the 7th edition of International Union Against Cancer tumor-node-metastasis classification. All specimens were snap-frozen in liquid nitrogen immediately after surgery, and then stored at -80 ℃ until analysis.

Cell lines and cell culture

The human GC cell lines MKN-28, SGC-7901 and MKN-45 and the normal gastric mucosal cell line GES-1 were obtained from Shanghai Institute of Digestive Disease (Shanghai, China). Cell lines were cultured in Dulbecco's modified Eagle's medium (Gibco, United States) with 10% fetal bovine serum (FBS, Gibco, United States).

Total RNA extraction and cDNA synthesis

Total RNA was extracted from the human tissues and GC cells using TRIzol Reagent (Invitrogen, United States) according to the manufacturer's instructions. The RNA concentration and purity were determined using the absorbance ratio at 260/280 acquired by a spectrophotometer. cDNA synthesis from 4 μg of total RNA was performed with a reverse transcription system kit (Promega, United States) according to the manufacturer's protocol. Briefly, samples were preincubated at 70 ℃ for 10 min, cooled on ice, then added to a reaction mixture consisting of 3 μL dNTP mixture, 2 μL M-MLV reverse transcriptase, 10 μL reverse transcription 5 × buffer, 1 μL Rnasin and 2 μL oligo-(dT)15 primer in a final volume of 20 μL. The reaction mixture was sequentially incubated at 95 ℃ for 15 min, 99 ℃ for 15 s and 62 ℃ for 40 s. The cDNA was

stored at -20 ℃ before use.

Quantitative real-time reverse transcription polymerase chain reaction assay

Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) was carried out in 96-well polypropylene microplates on an ABI Prism 7500 sequence detection PCR system (Applied Biosystems, United States) with the following primers: KLK12: 5'-GCCTCAACCTCTCCATCGTC-3' (forward) and 5'-CTTGAAGGACTCCCCCACAC-3' (reverse); β-actin: 5'-CATGTACGTTGCTATCCAGGC-3'(forward) and 5'-CTCCTTAATGTCACGCACGAT-3' (reverse). The 20 μL PCR reaction consisted of (2×) SYBR® Premix Ex Tag™ (Takara, Japan), 2 μL cDNA and 0.4 μL of each gene-specific primer. The thermal cycling protocol included an initial denaturation step at 95 ℃ for 30 s followed by 45 cycles at 95 ℃ for 5 s for denaturation and 62 ℃ for 40 s for primer annealing and extension. After the last cycle, a melting curve analysis was performed. All PCR reactions were run in triplicate. KLK12 mRNA expression was calculated from the standard curve, and quantitative normalization in each sample was performed using β-actin gene expression as an internal control. Relative quantification was performed using the $2^{-\Delta\Delta Ct}$ method.

Immunohistochemistry analysis

Immunohistochemistry (IHC) studies of hK12 were performed on surgical specimens from patients with GC using the avidin-biotin-peroxidase method (KIT-9702, Maixin Bio, China) on formalin-fixed, paraffin-embedded tissue specimens. All sections were incubated with polyclonal sheep anti-human KLK12 antibody (AF3095, R and D Systems, United States) at a dilution of 1:50. Slides were counterstained with hematoxylin. All sections were evaluated independently and blinded to outcome data by a pathologist three times. A cutoff of more than 30% of the tumor cells with moderate or strong KLK12 cytoplasmic staining in a gastric tumor section was considered to be positive.

siRNA transfection

The expression vector pBSKH1 (SIBS, China) was used for expression of siRNA. The siRNA designed to target the *KLK12* gene (sense strand: 5'-AAACAGUGACAGCCACGUATT-3', anti-sense strand: 5'-UACGUGGCUG UCACUGUUUGG-3') was inserted into the pBSKH1 vector according to the manufacturer's instructions, and then, the vector was transfected into the GC cells by the Lipofectamine™ 2000 (Invitrogen, United States). A mock vector-transfected clone of the cell line was used as a control. Stably transfected cell clones were tested for KLK12 expression by quantitative real-time RT-PCR and Western blotting.

Western blotting analysis

Cells were harvested 72 h after transfection, and whole-cell lysates were prepared for protein extraction. The protein concentrations of the samples were determined by the bicinchoninic acid protein assay. Proteins were separated in 10% sodium dodecyl sulphate polyacrylamide gels, electrophoretically transferred to polyvinylidenedifluoride membranes and incubated in a blocking solution of 5% skim milk powder for 1 h at room temperature. Membranes were then incubated with polyclonal sheep anti-human KLK12 antibody (1:500, AF3095, R and D Systems, United States) and anti-β-actin antibody (1:1000, sc-47778, Santa Cruz, United States) overnight at 4 ℃. The membranes were washed 3 times in tris-buffered saline tween-20 (TBST) and incubated for 1 h at room temperature with a 1:1000 dilution of secondary antibody conjugated to horseradish peroxidase (Invitrogen, United States). After incubation with a secondary antibody, the membranes were washed in TBST and developed using electrochemiluminescence according to the manufacturer's instructions.

In vitro cell proliferation assay

Cell proliferation was evaluated using the methylthiazolyltetrazolium (MTT) assay. Cells were grown in a monolayer culture and plated at a density of 4×10^3 cells/well into separate wells of a 96-well culture plate. The cells were incubated with MTT after 1, 2, 3, 4 or 5 d. Absorbance was measured at 570 nm using a microplate reader (SpectraMax 250, Molecular devices, United States). The experiments were performed in triplicate.

In vitro migration and invasion assays

The motility and invasiveness of plasmid-transfected cells were evaluated in 24-well transwell chambers with upper and lower culture compartments separated by polycarbonate membranes with 8-μm pores (CytoSelect™ 24 Well Cell Migration and Invasion Assay Combo Kit, 8-μm, CBA100-C, Cell Biolab, United States). Prior to plating cells into the transwells, the 24-well migration plate was allowed to warm up to room temperature for 10 min. A cell suspension containing 1.0×10^6 cells/mL in serum-free media was plated into the top chamber. Five hundred microliters of media containing 10% FBS was placed into the bottom chamber to act as a chemoattractant. Then, 300 μL of the cell suspension solution was added to the inside of each insert and incubated 24 h in a cell culture incubator. The cells that migrated through the 8-μm pores and adhered to the lower surface of the membrane were transferred to a clean well containing 400 μL of 0.09% crystal violet as a cell staining solution and washed several times in a beaker with water. The migratory cells were counted using light microscopy under high magnification, with 5 individual fields per insert. In a similar fashion, the invasiveness of plasmid-transfected cells was also evaluated using this kit (Invasion Chamber Plate, Cell Biolab, United States). Cells, media, experimental conditions, and the analysis performed were similar to those for the migration assays. Triplicate assays were performed for each group of cells in both the migration and invasion assays.

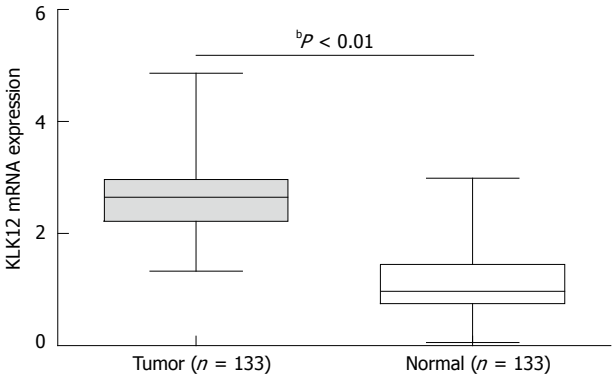

Figure 1 Upregulation of kallikrein 12 mRNA expression in gastric cancer. Quantitative real-time reverse transcription polymerase chain reaction showed that the mean expression value of kallikrein 12 (KLK12) mRNA in cancer tissues was significantly higher than the value in relevant normal tissues. Data are shown as mean ± SD, using the Student's *t* test ($^{b}P < 0.01$ between tumor and normal group, horizontal bars represent medians).

Statistical analysis

Statistical analysis were performed using SPSS 11.0 software (Shanghai Jiaotong University, China). The data are expressed as the mean ± SD. The Student's *t* test and one-way analysis of variance test were used to compare data between the different groups. The χ^2 test was used to assess the relationship between hK12 expression levels and clinicopathological characteristics of GC. Survival curves were drawn according to the Kaplan-Meier method, and the log-rank test was applied to compare the survival curves. Differences were considered significant at the $P < 0.05$ level.

RESULTS

KLK12 mRNA level is upregulated in GC patients

There were 133 GC patients included in the study. The age of the patients ranged from 18 to 87 years (median 61 years). Overall, 126 of the 133 patients (94.7%) showed a higher expression level of KLK12 mRNA in GC tissue specimens compared to noncancerous tissue specimens. The mean expression value of KLK12 mRNA in cancer tissues was significantly higher than the value in relevant normal tissues (2.75 ± 0.78 *vs* 1.10 ± 0.56, $P < 0.001$, Figure 1).

Immunostaining demonstrates hK12 overexpression in GC tissues

To further validate the expression and localization of hK12 in surgical specimens, IHC was performed on paraffin-fixed GC tissues and matched noncancerous mucosa of 133 patients. Dark-brown immunostaining was most prevalent in cancer cells, whereas the staining levels were lower in stromal cells or fibroblasts of GC tissues. hK12 expression was detected in 72.2% (96/133) GC samples with moderate or strong staining (Figure 2A), primarily located in the cytoplasm (Figure 2A and B). In contrast, negative immunostaining for hK12 was observed in normal gastric mucosal sections (Figure 2C and D).

Table 1 Relationship between human kallikrein 12 protein expression and clinicopathological features in 133 patients with gastric cancer

Feature	*n*	hK12 expression		*P* value[1]
		High (n = 96)	Low (n = 37)	
Sex				
Male	83	59	24	0.716
Female	50	37	13	
Age (yr)				
⩾ 61	69	47	22	0.277
< 61	64	49	15	
Depth of invasion (T)				
T1 + T2	21	14	7	0.539
T3 + T4	112	82	30	
Tumor size (cm)				
⩾ 5	77	59	18	0.180
< 5	56	37	19	
Lymph node metastasis				
Positive	95	76	19	0.001
Negative	38	20	18	
Lymphatic invasion				
Absent	47	33	14	0.708
Present	86	63	23	
Histological type				
Undifferentiated	94	77	17	< 0.001
Differentiated	39	19	20	
Stage				
Ⅰ + Ⅱ	44	25	19	0.005
Ⅲ + Ⅳ	89	71	18	

[1]*P* value was determined by the χ^2 test. hK12: Human kallikrein 12 protein.

Clinicopathological significance of hK12 expression in GC

The clinicopathological factors analyzed in relation to hK12 expression in tumor tissues are shown in Table 1. The incidence of lymph node metastasis was significantly higher ($P = 0.001$) in the high-expression group (76 of 95, 80.0%) than in the low-expression group (20 of 38, 52.6%). The histological type and pathological stage also correlated with the groups. However, no significant difference was observed with regard to sex, age, depth of invasion, tumor size or lymphatic invasion. The 5-year actuarial overall survival rates in patients with high hK12 levels and patients with low hK12 levels were 62.2% and 31.3%, respectively (Figure 3). The difference in survival rates between these two groups was statistically significant ($P = 0.002$).

Expression of KLK12 mRNA in GC cell lines

Furthermore, three well-characterized GC cell lines, MKN-28, SGC-7901 and MKN-45, and the normal gastric mucosal cell line GES-1 were chosen to examine KLK12 mRNA expression due to their diverse differentiation features. MKN-45 cells expressed the highest level of KLK12 across the four cell lines (Figure 4). Therefore, MKN-45 cells were chosen to do a series of functional experiments involving knockdown of KLK12 expression.

KLK12 siRNA-transfected GC cell line stably suppresses both KLK12 mRNA and hK12

As shown in Figure 5A, MKN-45 cells transfected with siRNA targeting KLK12 showed a remarkable decrease

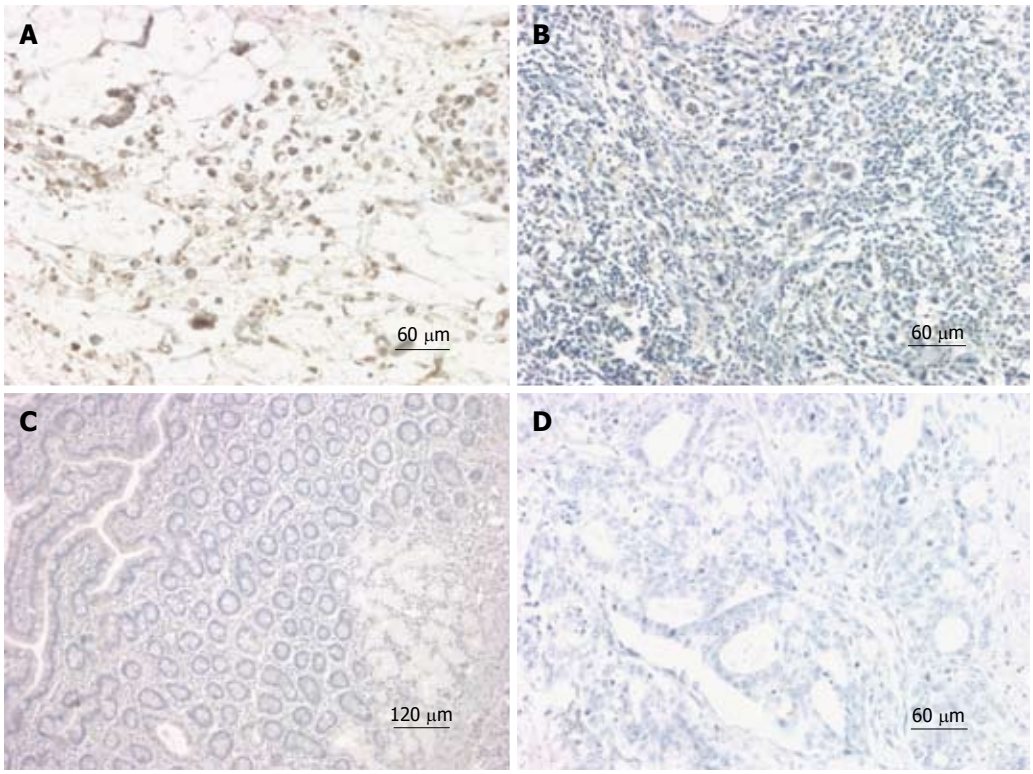

Figure 2 Expression of human kallikrein 12 protein in gastric cancer and non-cancerous mucosal tissues detected by immunohistochemistry. A: Strong positive human kallikrein 12 protein (hK12) immunostaining in gastric cancer (GC) tissues, hK12 staining was observed in the cytoplasm of cancer cells; B: Weak positive hK12 immunostaining in GC tissues; C: Negative hK12 immunostaining in relevant normal tissues; D: Negative hK12 immunostaining in GC tissues (original magnification A, B, D ×200, C ×100).

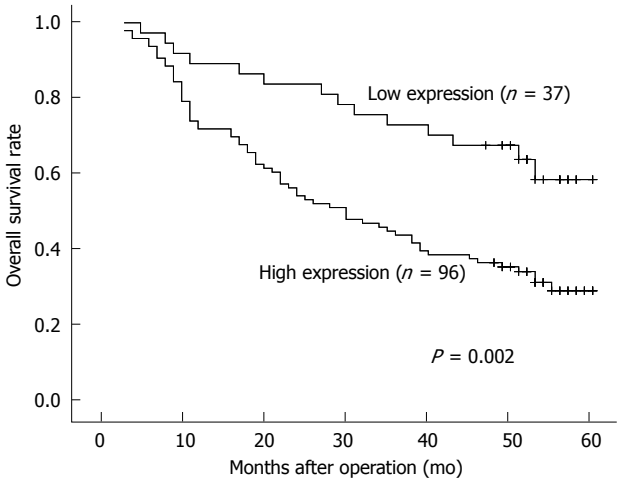

Figure 3 Overall survival of patients with gastric cancer according to human kallikrein 12 protein expression in the cancer tissues. Patients in the high human kallikrein 12 protein (hK12) expression group had a significantly poorer prognosis than those in the low hK12 expression group. Survival curves are drawn according to the Kaplan-Meier method, using the log-rank test to compare the survival rates (P = 0.002).

in the level of KLK12 mRNA compared to mock-transfected or parent MKN-45 cells, as determined by quantitative real-time RT-PCR. Furthermore, we performed Western blotting analysis to verify the efficiency of the KLK12 siRNA. The stable KLK12-suppressed clone was confirmed to express markedly lower levels (about one fourth) of hK12 than the MKN-45 parental cells (Figure 5B), confirming that KLK12 siRNA decreased KLK12 expression, consistent with the quantitative real-time RT-PCR results.

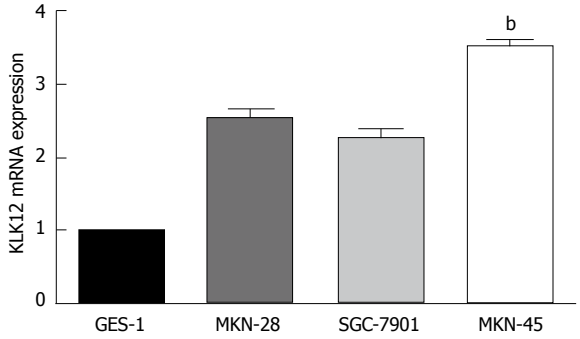

Figure 4 Expression of kallikrein 12 mRNA in gastric cancer cell lines and normal gastric mucosal cell line. Gastric cancer cell lines show higher levels of kallikrein 12 (KLK12) mRNA expression than normal gastric mucosal cell line, while MKN-45 cells expressed the highest level of KLK12 across the four cell lines. Data are shown as mean ± SD, using the one-way analysis of variance test ($^bP < 0.01$ *vs* other cell lines).

Knockdown of KLK12 affects the proliferative ability of MKN-45 GC cells

We analyzed whether suppressing the KLK12 expression would alter the growth rate of MKN-45 GC cells. As shown in Figure 6, transfection with KLK12 siRNA remarkably decreased the proliferative ability of MKN-45 cells when compared with the parent and mock-transfected cells (P = 0.001), especially from the 3rd to 5th days of the assay.

Knockdown of KLK12 affects the migratory ability of MKN-45 GC cells

We evaluated whether suppression of KLK12 expression could alter the ability of *in vitro* migration and invasion

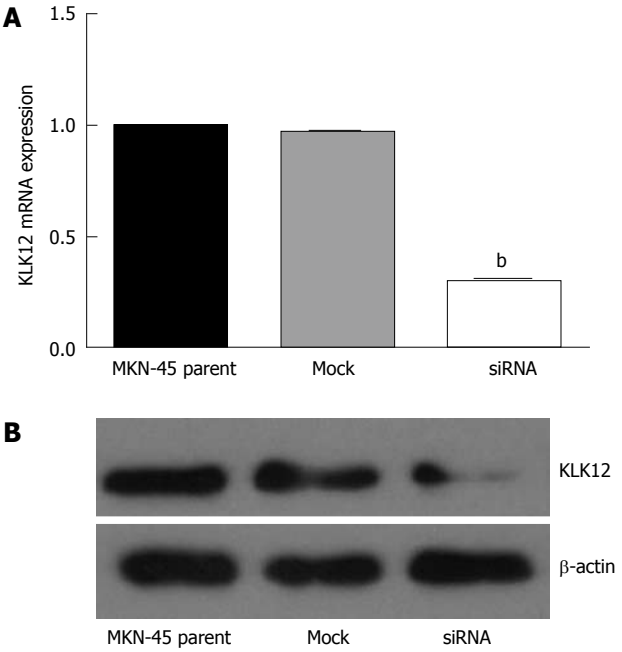

Figure 5 Efficiency of small interfering RNA in silencing the kallikrein 12 mRNA, and protein expression in MKN-45 cells. A: MKN-45 cells transfected with small interfering RNA targeting human kallikrein 12 (*KLK12*) gene showed a remarkable decrease in the level of KLK12 mRNA compared to mock-transfected or parent MKN-45 cells. Data are shown as mean ± SD, using the one-way analysis of variance test ($^bP < 0.01$ *vs* other cell lines); B: Western blotting analysis showed a reduced protein expression in KLK12 small interfering RNA (siRNA) transfected cells. The protein levels are measured by Image J software (National Institutes of Health, United States) with β-actin protein normalization.

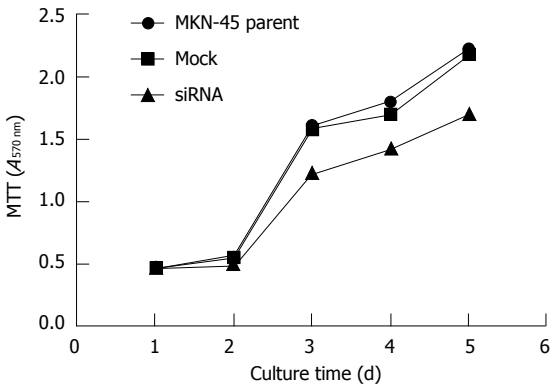

Figure 6 Reduction of cell proliferation by methylthiazolyltetrazolium assay after silencing the kallikrein 12. The proliferative ability significantly decreased in MKN-45 cells after transfection with kallikrein 12 small interfering RNA (siRNA), especially from the 3rd to 5th days of the assay. Data are shown as mean ± SD, using the one-way analysis of variance test ($P = 0.001$). MTT: Methylthiazolyltetrazolium.

of MKN-45 cells. As shown in Figure 7, fewer KLK12 siRNA-transfected cells migrated through the chambers (22.00 ± 1.81) when compared to the parent (46.47 ± 2.42) or mock-transfected (45.40 ± 1.99); these differences were statistically significant ($P < 0.001$, Figure 7A and B). However, in the invasion test, the number of KLK12 siRNA-transfected cells that invaded the chambers in 18.40 ± 1.12, closely similar to both the parent (18.67 ± 0.98) and mock-transfected cells (18.53 ± 0.92). There was no significant difference between the three groups in the invasion assay ($P = 0.054$).

DISCUSSION

The *KLK12* gene is a new member of the *KLK* gene family, some members of which are implicated in the initiation and progression of cancer[20-22]. KLK12 is encoded by 5 coding exons, and is structurally similar to serine proteases and other known KLKs. KLK12 is expressed in a variety of tissues including the salivary gland, stomach, uterus, lung, thymus, prostate, colon, brain, breast, thyroid, and trachea. Initially, it was reported that expression of KLK12 is downregulated at the mRNA level in breast cancer tissues and is upregulated by steroid hormones in breast and prostate cancer cell lines[17]. Memari *et al*[19] demonstrated that more than 95% of prostate cancers were KLK12 positive in a tissue microarray study. To the best of our knowledge, this study is the first investigation to analyze KLK12 expression in GC. We performed quantitative real time RT-PCR, IHC and a series of functional assays utilizing siRNA-mediated downregulation of KLK12 expression to determine the role and explore the mechanism of KLK12 in GC. Our results showed a drastic difference in KLK12 expression between GC and normal mucosal tissues. Substantially elevated expression of the *KLK6* gene has been observed in clinical tissue samples and several GC cell lines[13], while other authors have reported that cancerous stomach tissues were found to present significantly decreased levels of KLK11 and KLK13 mRNA transcripts in comparison with their normal counterparts[15,16]. Moreover, our study showed that high hK12 expression was significantly correlated with the lymph node metastasis ($P = 0.001$), histological type ($P < 0.001$) and pathological stage ($P = 0.005$) of GC. High expression of hK12 was also associated with a poor prognosis for patients with GC. These findings suggest that enhanced expression of KLK12 might play an important role in various pathological processes of GC. MKN-45, a poorly differentiated GC cell line, was chosen for functional experiments because KLK12 mRNA expression was higher in MKN-45 cells compared to other cell lines. Additionally, hK12 expression was significantly higher in undifferentiated GC. In our experiments, we established clones with suppressed KLK12 expression by gene silencing using RNA interference (RNAi) techniques. RNAi is mediated by siRNAs that are produced from long double stranded RNAs of exogenous or endogenous origin by an endonuclease of the RNase-Ⅲ type called Dicer[23]; this technique has emerged as a powerful tool for understanding gene functioning. As quantitative real-time RT-PCR and Western blotting revealed, KLK12 siRNA remarkably reduced KLK12 expression of MKN-45 cells. MTT proliferation assays showed with KLK12 siRNA dramatically decreased the proliferation of MKN-45 cells when compared with MKN-45 parent and mock-transfected cells. The differences were significant, especially from the 3rd day to the 5th day after transfection, which is in accor-

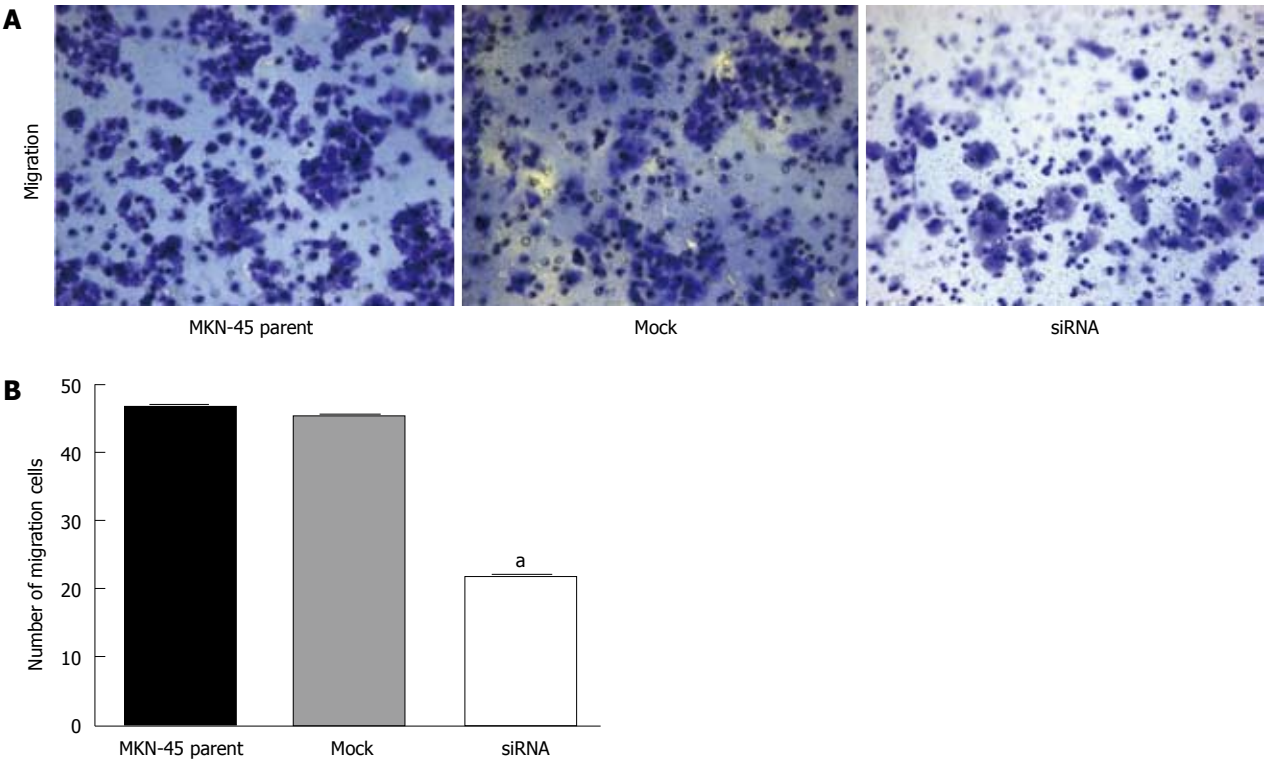

Figure 7 Effect of cell migration by kallikrein 12 knockdown. A: 24-well transwell chambers with upper and lower culture compartments separated by polycarbonate membranes with 8-μm pores were used for migration or invasion assay. The chambers were stained by 0.09% crystal violet and cells were counted using light microscopy under high magnification (magnification ×10); B: The migration or invasion cells were counted in 5 individual fields per insert. Values were the number of cells. Data are shown as mean ± SD, using the one-way analysis of variance test ($^aP < 0.05$ *vs* other cell lines).

dance with the Western blotting data showing knockdown of KLK12 expression was greatest 72 h after transfection. Metastasis and local recurrence are known to be the primary causes of death after radical surgery of GC patients. Tumor metastasis is a complicated process and involves many steps including migration of cancer cells to, and invasion through, the basement membrane[24]. In our experiments, significantly fewer KLK12-suppressed cells migrated across polycarbonate membranes when compared to MKN-45 parent and mock-transfected cells ($P < 0.001$). The invasiveness of all three cell lines was comparable, and the KLK12-suppressed cells displayed no difference in the invasion assay compared to parent or mock-transfected cells. Other KLKs have been shown to degrade components of the extracellular matrix. Magklara *et al*[25] reported that hK6 can degrade fibrinogen and collagen type I , basic constituents of the extracellular matrix, as well as collagen type Ⅳ, a major component of the basement membrane *in vitro*. The lysis of certain components of the extracellular matrix is linked with an altered regulation of tumor metastasis. However, Memari *et al*[26] reported that KLK12 is secreted as an inactive proenzyme, which is able to autoactivate to gain enzymatic activity. However, active KLK12 quickly loses its activity due to autodegradation, and its activity can also be rapidly inhibited by zinc ions and by alpha2-antiplasmin through covalent complex formation. According to these results, it is reasonable to conclude that the increased KLK12 expression may not play an important role in metastasis. In conclusion, we present an early report that KLK12 was remarkably overexpressed in GC tissues and that high KLK12 expression levels were associated with the lymph node metastasis, histological type, pathological stage and poor patient prognosis. Our findings also demonstrate that knockdown of KLK12 expression leads to reduced proliferation and migratory ability with little effect on invasiveness in MKN-45 GC cells. Consequently, KLK12 might serve as a novel diagnostic and prognostic biomarker, as well as a potential therapeutic target, in GC.

COMMENTS

Background

Gastric cancer (GC) is the fourth most common malignancy and the second most common cause of cancer mortality worldwide. Although morbidity and mortality rates for GC are deceasing steadily in many countries, the overall outcome for patients with GC has not changed significantly in recent decades. It is necessary to find a reliable biomarker for GC to be incorporated into routine clinical practice.

Research frontiers

The human kallikrein 12 (*KLK12*) gene is a new member of the *KLK* gene family. Similar to other kallikreins, KLK12 is a proteolytic enzyme with serine protease activity, and participates in several biological processes. Moreover, KLK12 may also play a role in human carcinogenesis of cancers such as breast and prostate cancer. However, little is known about KLK12 in human GC.

Innovations and breakthroughs

The results of this study provide strong evidence suggesting that KLK12 expression is upregulated in GC tissue and correlated with lymph node metastasis, poor histological type, advanced clinical stage and decreased overall survival rate. Furthermore, the authors found that knockdown of KLK12 markedly

decreased the proliferative and migratory abilities of MKN-45 GC cells.

Applications

These results demonstrate that KLK12 might serve as a novel diagnostic and prognostic biomarker in GC. This study may represent a future strategy for therapeutic intervention in the treatment of patients with GC.

Terminology

The *KLK* genes are a newly identified subgroup of putative serine proteases, consisting of 15 genes located within approximately 256 kb on chromosome 19q13.3-4.

Peer review

This is a good descriptive study in which the authors investigate expression of KLK12 in GC tissues. The biological effects of KLK12 knockdown in the GC cell line MKN-45 were also studied. The results are interesting and suggest knockdown of KLK12 overexpression in GC may be a potential therapeutic target. The study was well designed and the data are convincing.

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S- Editor Gou SX **L- Editor** A **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v18.i45.6605

World J Gastroenterol 2012 December 7; 18(45): 6605-6613
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Hepatoprotective effects of baicalein against CCl_4-induced acute liver injury in mice

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Author contributions: Huang HL and Wang YJ contributed equally to the manuscript; Zhu RZ designed the research; Huang HL, Wang YJ, Zhang QY, Liu B, Wang FY and Li JJ performed the research and analyzed the data; Zhu RZ wrote the paper.
Supported by The Fundamental Research Funds for the Central Universities No. JKQ2011008, JKQ2011010; Postdoctoral Science Foundation of Jiangsu Province, China, No. 1101029C
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Received: June 28, 2012 Revised: September 6, 2012
Accepted: September 29, 2012
Published online: December 7, 2012

Abstract

AIM: To investigate the hepatoprotective effect of baicalein against carbon tetrachloride (CCl_4)-induced liver damage in mice.

METHODS: Mice were orally administered with baicalein after CCl_4 injection, and therapeutic baicalein was given twice a day for 4 d. The anti-inflammation effects of baicalein were assessed directly by hepatic histology and serum alanine aminotranferease and aspartate aminotransferase measurement. Proliferating cell nuclear antigen was used to evaluate the effect of baicalein in promoting hepatocyte proliferation. Serum interleukin (IL)-6, IL-1β and tumor necrosis factor-α (TNF-α) levels were measured by enzyme-linked immunosorbent assay and liver *IL-6*, *TNF-α*, transforming growth factor-α (*TGF-α*), hepatocyte growth factor (*HGF*) and epidermal growth factor (*EGF*) genes expression were determined by quantitative real-time polymerase chain reaction.

RESULTS: CCl_4-induced acute liver failure model offers a survival benefit in baicalein-treated mice. The data indicated that the mRNA levels of IL-6 and TNF-α significantly increased within 12 h after CCl_4 treatment in baicalein administration groups, but at 24, 48 and 72 h, the expression of IL-6 and TNF-α was kept at lower levels compared with the control. The expression of TGF-α, HGF and EGF was enhanced dramatically in baicalein administration group at 12, 24, 48 and 72 h. Furthermore, we found that baicalein significantly elevated the serum level of TNF-α and IL-6 at the early phase, which indicated that baicalein could facilitate the initiating events in liver regeneration.

CONCLUSION: Baicalein may be a therapeutic candidate for acute liver injury. Baicalein accelerates liver regeneration by regulating TNF-α and IL-6 mediated pathways.

Key words: Baicalein; Carbon tetrachloride; Liver injury; Liver regeneration; Hepatocyte proliferation

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Huang HL, Wang YJ, Zhang QY, Liu B, Wang FY, Li JJ, Zhu RZ. Hepatoprotective effects of baicalein against CCl_4-induced acute liver injury in mice. *World J Gastroenterol* 2012; 18(45): 6605-6613 Available from: URL: http://www.wjgnet.

com/1007-9327/full/v18/i45/6605.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i45.6605

INTRODUCTION

Liver is an important organ which plays a central role in metabolic homeostasis[1]. It also has an amazing regenerative capability after liver mass loss, as demonstrated by Higgins *et al*[2] in 1931. Carbon tetrachloride (CCl_4)-induced hepatic injury is a very classic model widely used for hepatoprotective drug screening[3,4]. The acute hepatotoxicity of CCl_4 lies in its biotransformation to trichloromethyl free radical (CCl_3) or trichloroperoxyl radical ($CCl_3O_2^-$) produced by the mixed-function cytochrome P450 oxygenase system of the endoplasmic reticulum, which causes oxidative stress and membrane damage[5]. These free radicals cause lipid peroxidation which results in hepatocellular damage and enhances formation of inflamed tissues. The advantage of this model is that CCl_4 can fulminate hepatitis within a few hours, which specifically leads to necrosis and fatty liver, in a similar way as what happens in the cases of acute hepatitis. Meanwhile, following an inflammatory response launched by resident inflammatory cells, CCl_4-induced acute liver injury also involves an intricately regulated process of hepatocyte regeneration when the dosage of CCl_4 is below lethal level which would lead to irreversible liver damage[6,7].

Baicalein (5, 6, 7-trihydroxyflavone, BAE, $C_{15}H_{10}O_5$) is a flavonoid extract from the root of *Scutellaria baicalensis Georgi*, a plant used in traditional Chinese medicine. Previous studies reported that baicalein has multiple functions. It acts as an anti-bacteria and anti-inflammation agent, inhibits the aggregation of blood platelets, decreases the production of endotoxin, and alleviates the reperfusion injury in ischemic tissues[8,9]. Baicalein was indicated to suppress the growth of human hepatoblastoma cells[10,11], human breast cancer cells[12,13], human lung fibroblasts and peripheral lymphocytes[14] and human leukemia HL-60 cells[15]. Baicalein has beneficial effects against the cytotoxicity and genotoxicity to hepatocytes by tert-butylhydroperoxide *via* quench free radicals. Moreover, baicalein could protect animals from *D*-galactosamine/lipopolysaccharides induced acute liver failure in murine models, and especially reduce apoptosis (even hepatic necrosis) *via* cellular FLICE-like inhibitory protein and mitogen-activated protein kinase pathway[16,17]. However, the antihepatotoxic mechanism of baicalein remains vague so far. The aforementioned investigations for liver diseases on the role of baicalein in selectively inducing apoptosis of cancer cells and inhibiting normal hepatocyte apoptosis, prompted us to study whether baicalein would increase the secretion of various inflammatory cytokines and facilitate regeneration of liver cells. The aim of this study is to assess whether baicalein could prevent acute liver injury induced by CCl_4 in mice and to investigate the possible mechanism of its protective role.

MATERIALS AND METHODS

Animals and chemicals

Specific pathogen-free male C57 BL/6 mice (8 wk old) were obtained from Shanghai Slac Laboratory Animal Corporation. The mice were maintained in a conventional clean facility in accordance with the National Animal Care and Use Committee. CCl_4 and baicalein were purchased from Sigma-Aldrich Biotechnology (St Louis, MO, United States). Assays kits for the detection of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were purchased from Jiancheng Biological Technology, Inc. (Nanjing, China). Mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA) and the SABC Staining Kit were from Boster Biological Technology (Wuhan, China). Serum levels of interleukin (IL)-1β, IL-6 and tumor necrosis factor-α (TNF-α) were measured by enzyme-linked immunosorbent assay (ELISA) kits for IL-1β, IL-6 and TNF-α from R and D system (Minneapolis, MN, United States). All other chemicals were of the highest grade commercially available.

Induction of liver injury and baicalein administration

Acute liver injury in mice was induced by intraperitoneal injection of CCl_4 at a dose of 1 mL/kg body weight (1:3 diluted in corn oil). A lethal dose was administered by intraperitoneal injection of CCl_4 at 2.6 mL/kg (1:1 diluted in corn oil). At the indicated time points, serum and liver specimens were collected. Mice were orally administered with baicalein (80 mg/kg) dissolved in CMC-Na to 200 mg/L 1 h after CCl_4 injection, and the same dose of baicalein was given twice a day for 4 d, and control mice were treated with same dosage CMC-Na.

Serum AST and ALT

Serum AST and ALT levels were determined with a commercial assay kit (Nanjing Jiancheng Biological Technology, Inc., China). Enzyme activities were presented in international unit per liter (IU/L).

Histology-injury grading

Formalin-fixed, paraffin-embedded liver sections were stained with hematoxylin-eosin for the histological studies. To evaluate the degree of necrosis after acute liver injury, we created an injury grading score (Grades I -Ⅳ) based on severity of necrotic lesions in the liver parenchyma (Table 1).

Proliferating cell nuclear antigen staining

For PCNA immunohistochemical staining, de-paraffinized sections of liver blocks were used. Liver tissues were fixed for 24 h in neutral buffered formalin, processed routinely and embedded in wax. Immunohistochemical staining was performed as previously described[18]. The sectioned liver tissues were stained using a mouse monoclonal antibody against PCNA and the SABC Staining Kit (Wuhan Boster Biological Technology, Wuhan, China) according to the

Table 1 Liver injury grading system

No. of mice	Day 2[1]	Day 3[1]	Day 5[1]	Day 7[1]
Baicalein				
1	Ⅲ	Ⅰ	Ⅰ	0
2	Ⅲ-Ⅳ	Ⅱ	0	0
3	Ⅲ	Ⅰ	0	0
4	Ⅲ	Ⅱ	0	0
5	Ⅲ-Ⅳ	Ⅰ	Ⅰ	0
6	Ⅲ	Ⅰ	0	0
Control				
1	Ⅳ	Ⅱ-Ⅲ	Ⅰ-Ⅱ	0
2	Ⅲ-Ⅳ	Ⅲ	Ⅱ	Ⅰ
3	Ⅲ-Ⅳ	Ⅲ	Ⅱ	Ⅱ
4	Ⅳ	Ⅲ	Ⅱ	0
5	Ⅳ	Ⅱ	Ⅱ	Ⅰ
6	Ⅳ	Ⅲ	Ⅰ	Ⅰ

[1]Days after CCl4 treatment at the sacrifice point. Injury grading with respect to severity of necrosis in liver parenchyma. Grade 0: Normal histology; Grade Ⅰ: Presence of degenerated hepatocytes with only rare foci of necrosis; Grade Ⅱ: Mild centrilobular necrosis around the central vein, occupying only a part of Rappaport's zone Ⅲ; Grade Ⅲ: Established necrosis limited to zone Ⅲ; Grade Ⅳ: Extensive, confluent centrilobular necrosis involving Rappaport's zone Ⅲ and Ⅱ.

manufacturer's protocol, then subjected to photomicroscopic observation (NIS-Elements Basic Research, Nikon Eclipse 50i, Kanagawa, Japan).

ELISA

Serum IL-1β, IL-6 and TNF-α levels were measured by ELISA kit (RD system, Minneapolis, MN, United States) according to the manufacturer's instructions. Cell lysates were generated by adding 1 mL fresh medium to 100 mg liver specimen or 1×10^7 cells followed by three freeze-thaw cycles. Transforming growth factor-α (TGF-α), hepatocyte growth factor (HGF) and epidermal growth factor (EGF) and ELISA kits were used to determine protein concentrations[19,20]. ELISA was performed in triplicate for each sample of lysate.

Real-time quantitative polymerase chain reaction

Total RNA was obtained from the liver of mice and was prepared using TRIzol reagent (Invitrogen, Carlsbad, CA, United States). The quantification and qualification of RNA were performed using ultraviolet absorbance assay and electrophoresis in 1.2% agarose. RNA quality was satisfactory for the 28s rRNA band on gel and had twice the intensity of the 18s rRNA band without significant smearing of rRNA. Real-time quantitative polymerase chain reactions (PCRs) were performed with the MJ chromo 4 reverse transcription-PCR detection system (Bio-Rad Laboratories, Hercules, CA, United States). Specific primers were designed using Primer 5.0 software (Premier Biosoft International, Palo Alto, CA, United States) and their sequences are listed in Table 2. As an internal control, the expression of the housekeeping gene *β-actin* was measured and remained constant at all the experimental conditions studied.

Table 2 Primer sequences used for real-time quantitative polymerase chain reaction

Gene	Sense	Anti-sense
IL-6	CCACTCCCAACAGACCT-GTCTATAC	CACAACTCTTTTCTCATTTC-CACGA
TNF-α	AAGCCTGTAGCCCACGTC-GT	CGTAGTCGGGGCAGCCTT-GTC
HGF	GTGCTGGGCATTACTAT-GATGG	CTGCATCTCCCTCTCACACG
TGF-α	GGCGGCTGCAGTGGT-GTCTC	AGCCACCACAGCCAGGAG-GTHGF
EGF	CGGACAGCTACACGGAATG	CGAGGCAGACACAAATA-ACCC
β-actin	AGCCTTCCTTCTTGGGTATG	GTGTTGGCATAGAGGTCTT-TAC

TNF-α: Tumor necrosis factor-α; IL: Interleukin; TGF-α: Transforming growth factor-α; HGF: Hepatocyte growth factor; EGF: Epidermal growth factor.

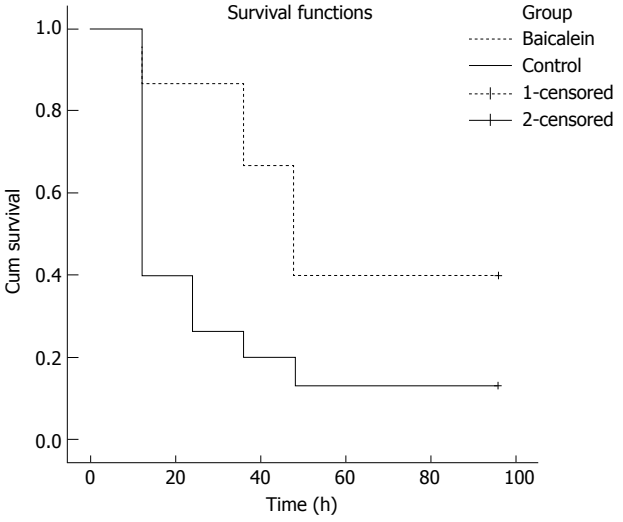

Figure 1 Baicalein increased probability of survival after a lethal dose of carbon tetrachloride (2.6 mL/kg). Mice (n = 15) were administered with or without baicalein twice a day for 5 d. Survivals were scored twice a day, and the results were analyzed using the log-rank test and expressed as the Kaplan-Meier survival curves. P = 0.009 between control and baicalein groups.

Statistical analysis

Student's t test (unpaired, two-tailed) was used for comparisons between data from specified different conditions. Results from survival experiments were analyzed using the log-rank test and presented as Kaplan-Meier survival curves.

RESULTS

Baicalein reduces mortality after a lethal dose performance

In a previous experiment to observe the dosage-dependent effect of CCl_4, we found that 2.6 mL/kg CCl_4 was a median lethal dose (a mortality of 50%, data not shown) within 24 h. Oral baicalein administration offers a survival

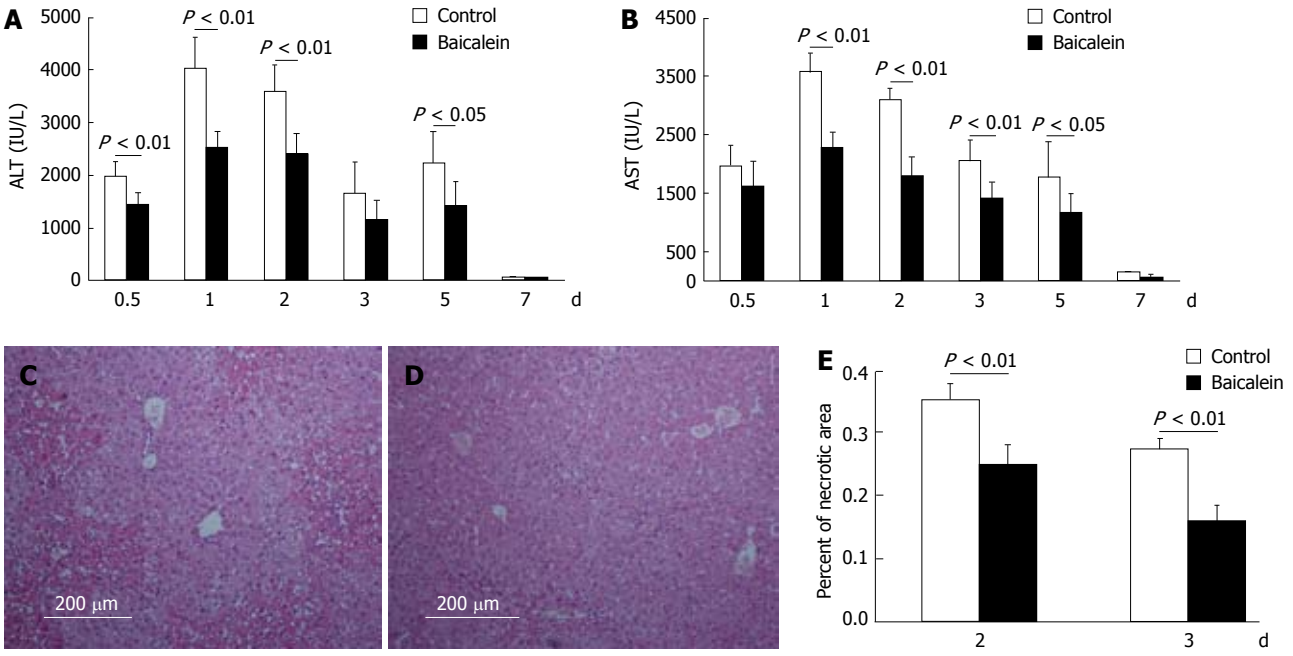

Figure 2 Baicalein protects liver against carbon tetrachloride induced acute liver injury. A: Serum alanine aminotransferase (ALT); B: Serum aspartate aminotransferase (AST); C: Hematoxylin and eosin (HE) stained liver sections of control group 3 d after carbon tetrachloride (CCl_4) treatment; D: HE stained liver sections of baicalein group 3 d after CCl_4 treatment; E: Percent of necrotic areas in control group and baicalein group 2 and 3 d after CCl_4 treatment. Mice received intraperitoneal CCl_4 at the dosage of 1 mL/kg body weight (1:3 diluted in corn oil). Mice in baicalein group were orally administered baicalein (80 mg/kg) 1 h after CCl_4 injection, twice a day for 4 d (original magnification, ×100). Necrosis with clusters of inflammatory cells around central vein was seen in control group; and histological recovery with only inconspicuous necrosis remaining around central vein, and very few inflammatory cells were present in the baicalein group. Control mice were treated with an equal volume of CMC-Na. Values represent mean ± SE (n = 6). $P < 0.05$, $P < 0.01$ between control and baicalein groups.

benefit for mice, increasing the probability of survival significantly one d after CCl_4 injection ($P = 0.009$, Figure 1).

Baicalein protects mice from acute hepatocellular damage

To confirm the effect of bacailein in protecting mice from hepatic damage, we used serum ALT and AST levels as indicators for liver injury. In the control group, the serum level of ALT and AST rapidly reached the peak level at day 1, and decreased thereafter, while baicalein significantly inhibited the elevated ALT and AST from day 1 to day 5 (n = 6) (Figure 2A and B). The attenuated increase of serum AST and ALT indicated that bacailein plays a direct protective role in hepatocytes. To evaluate the effect of baicalein on hepatocellular necrosis and inflammation, histological changes in the liver after CCl_4 administration with or without baicalein treatment were examined by histology-injury grading (Table 1). Liver sections from the baicalein-treated mice demonstrated only moderate necrosis involving the centrilobular areas, maintaining a rather normal architecture. The necrotic areas were significantly diminished around the central vein and centrilobular regions in bacailein-treated mice at day 3 (Figure 2C-E). These findings indicated that bacailein has potential antihepatotoxic activity.

Baicalein promotes hepatocyte proliferation from an early phase

To confirm whether baicalein has the potent advantage of accelerating hepatocyte proliferation from an early phase, we investigated the proliferation of hepatocytes using immunostaining of PCNA in sections of liver tissue at days 2 and 3. The PCNA staining confirmed that baicalein administration increased the number of positive staining cells more significantly at day 2 compared with the control group (Figure 3A and B). A great number of $PCNA^+$ hepatocytes could be detected in the liver sections of baicalein-treated mice at day 3 (Figure 3C and D), which demonstrated that baicalein significantly increased the number of $PCNA^+$ cells. Numbers of $PCNA^+$ cells in at least 12 mm^2 tissue sections were counted for each mouse, and data showed that bacailein could accelerate hepatocyte proliferation (Figure 3E).

Serum levels of IL-1β, IL-6 and TNF-α

To evaluate the hepatoprotective mechanism of baicalein, serum IL-1β, TNF-α and IL-6 levels were determined by ELISA kit. Serum IL-1β was found to be elevated after CCl_4 treatment[21], whereas baicalein administration resulted in significant attenuation of the elevation (Figure 4A). CCl_4-induced acute liver injury could activate hepatic non-parenchymal cells (including Kupffer cells and stellate cells) and increase the production of TNF-α and IL-6[22]. In our study, we found that serum TNF-α and IL-6 were rapidly increased and reached the peak level within 12 h in baicalein administration group as compared with the control group, and then decreased within 24 h (Figure 4B and C).

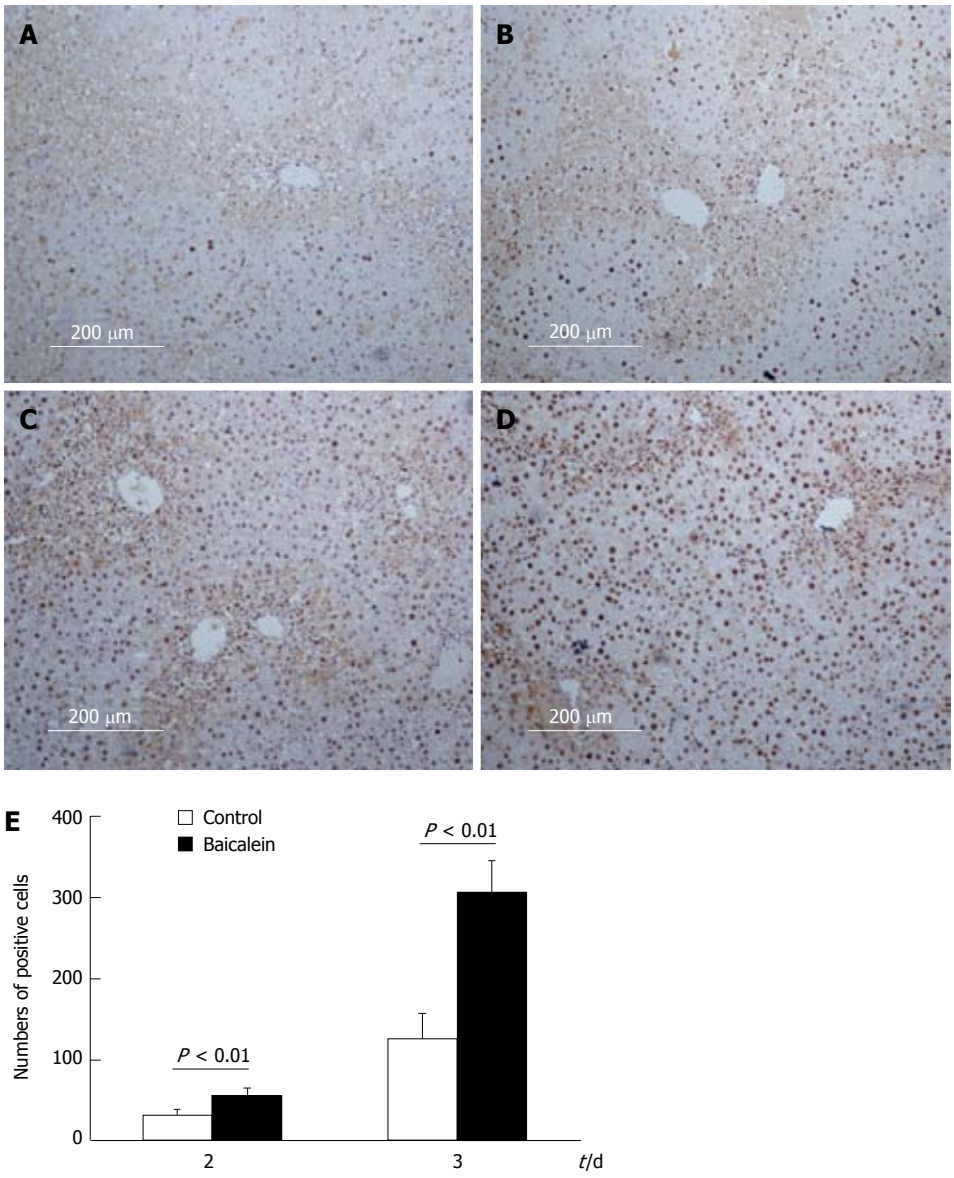

Figure 3 Proliferation status of carbon tetrachloride induced mice after treated with or without baicalein. A, B: Immunostaining of proliferating cell nuclear antigen (PCNA) in liver sections from control (A) and baicalein (B) groups 2 d after carbon tetrachloride (CCl_4) treatment; C, D: Immunostaining of PCNA in liver sections from control (C) and baicalein (D) groups 3 d after CCl_4 treatment; E: Numbers of $PCNA^+$ cells in CCl_4 induced mice after treated with or without baicalein. At least six 12-mm^2 tissue sections were counted for each mouse. Values represent mean ± SE (n = 6). $P < 0.01$ between control and baicalein groups.

Expression of TNF-α and IL-6 in liver

Real-time quantitative PCR was used to quantify the expression of TNF-α and IL-6 in mouse liver. Data showed that in baicalein administration group, the production of *TNF-α* and *IL-6* mRNA reached a peak level, which was even higher than in the control group, and then decreased rapidly in 24 h (Figure 5A and B).

Expression of TGF-α, HGF and EGF in liver

Real-time quantitative PCR was used to quantify the levels of TGF-α, HGF and EGF mRNA in liver. Data showed that the production of TGF-α, HGF and EGF mRNA was upregulated more rapidly in the baicalein administration group during the early phase and kept at a generally higher level within the process of liver regeneration (Figure 5C-E).

DISCUSSION

The model of acute intoxication with CCl_4 has been used for decades to investigate the response of acute liver injury, because the elementary lesions caused by this hepatotoxin replicate those seen in most cases of human liver diseases, which makes it a good model to study both signal transduction and cell cycle events *in vivo*[23,24]. Using this delicate model, we have identified the protective effect of baicalein against the typical acute liver injury.

Oral administration of baicalein to mice which have received a LD_{50} dosage of CCl_4 resulted in a significantly reduced mortality rate. Since the pathological effect of CCl_4 in the animals has been proved to be mainly restricted to the liver and lethality of high-dose CCl_4 is mostly related with organ failure following acute liver failure instead of direct injury to other organs, it is reasonable to hypothesize that administration of baicalein can reduce animal mortality mainly through attenuating acute liver damage by CCl_4, and facilitating the preservation and restoration of liver functions.

It has been proved that baicalein administration indeed attenuated acute liver damage. The indicators for

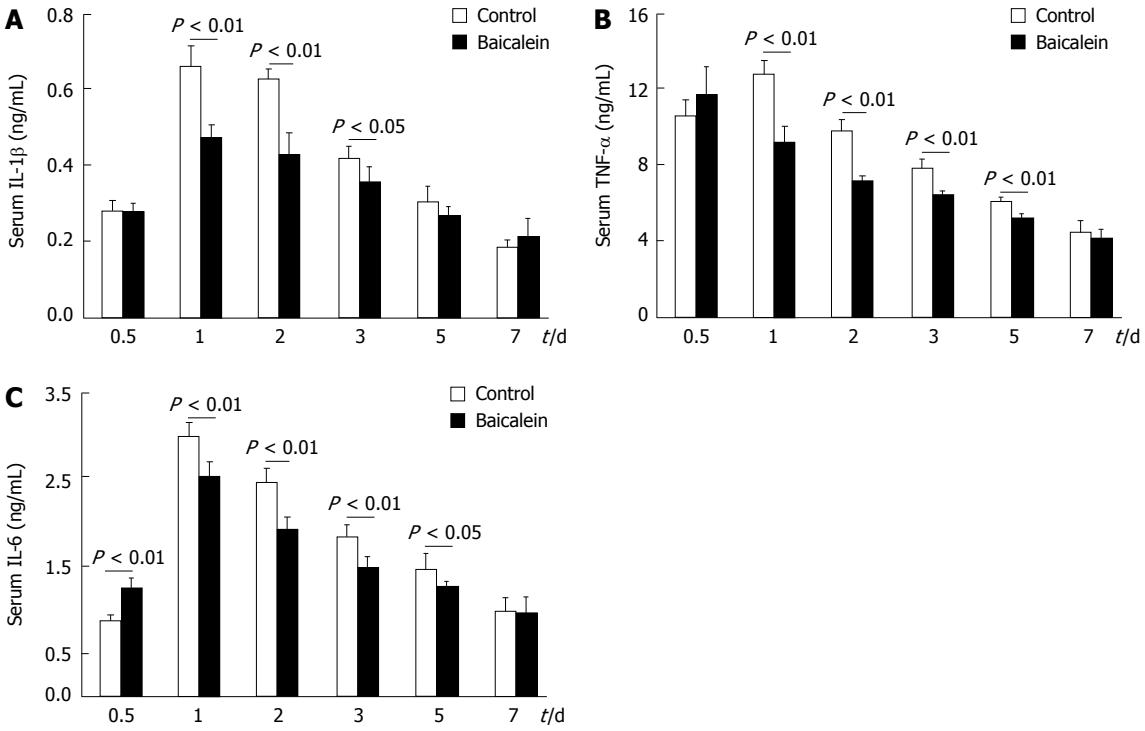

Figure 4 Levels of interleukin-1β, interleukin-6 and tumor necrosis factor-α in serum in control and baicalein groups after CCl_4 (1 mL/kg) treatment. A: Serum interleukin (IL)-1β; B: Serum tumor necrosis factor-α (TNF-α); C: Serum IL-6. A, B and C were determined by enzyme-linked immunosorbent assay kit. Values represent mean ± SE (n = 6). $P < 0.05$, $P < 0.01$ between control and baicalein groups.

the liver damage we have utilized are serum aminotransferase activities, including AST and ALT. They are commonly referred to as "liver enzymes", because the levels of these enzymes are released from damaged hepatocytes into the blood, and their levels in the serum have been widely recognized as a very important indicator to judge the severity of acute hepatic injury[25]. In our experiment, administration of baicalein attenuated the elevated serum ALT and AST induced by CCl_4 in mice, which indicated that the proportion of damaged hepatocytes was reduced as a direct result of baicalein administration. Elevated ALT level was found to be significantly attenuated 12 h after CCl_4 treatment, while similar phenomenon appeared at 24 h after CCl_4 treatment for AST. Both time points are defined as the early-stage liver damage in which cell apoptosis and necrosis dominate the process. When the liver damage progresses over time, the speed of cell damage as a result of either cell apoptosis or necrosis is reduced, as indicated by the relative decrease of AST/ALT levels at later time points of days 3 to 5. On the other hand, regeneration of liver gradually took place from the middle to late stages of liver damage, during which cell proliferation rate would naturally increase till the original weight and shape of the liver and its functions, is restored. We used another statistical index to measure the possible role of baicalein in the regeneration of liver tissue. It is the density of positive cells in a certain area of tissue section immunostained with PCNA antibody. The index strongly indicated that baicalein treatment contributes to a faster liver recovery after CCl_4-induced liver injury by promoting the endogenous regeneration process from the middle stage of the entire liver damage process. We also used histological methods as supportive means to reveal the degree of cell necrosis and inflammation. Data also showed that oral baicalein administration inhibited inflammation, necrosis, and destruction of liver architecture.

To investigate the underlying mechanism, we evaluated the effects of baicalein treatment on the serum level of certain key cytokines tightly related to inflammation and cell proliferation. IL-1β, IL-6 and TNF-α, as acute-phase proteins, are considered to be the special biomarkers that reflect inflammatory status[26]. IL-1β plays a key role in inflammation, usually leading to tissue destruction. Furthermore, IL-1β has been previously shown to antagonize hepatocyte proliferation[27,28]. Serum IL-1β can increase dramatically during different inflammatory and no-ninflammatory processes. In the present study, we observed that baicalein administrated mice demonstrated a significantly lower serum level of IL-1β at days 1, 2, 3 and 5, compared with the control group. The decreased level of inflammatory cytokines may explain the accelerated liver regeneration observed in baicalein administrated mice. IL-6 and TNF-α expression has been identified as attractive targets for liver regeneration. The release of TNF-α, as a pro-inflammatory mediator in liver apoptosis, is also linked to cytotoxicity induced by CCl_4[17,29]. Kupffer cells (macrophages in liver) produce TNF-α in rapid response to tissue injury, which then up-regulates the expression of IL-6. TNF-α and IL-6 together activate the neighboring hepatocytes, leading to signal transducer and activator of transcription STAT3 activation and

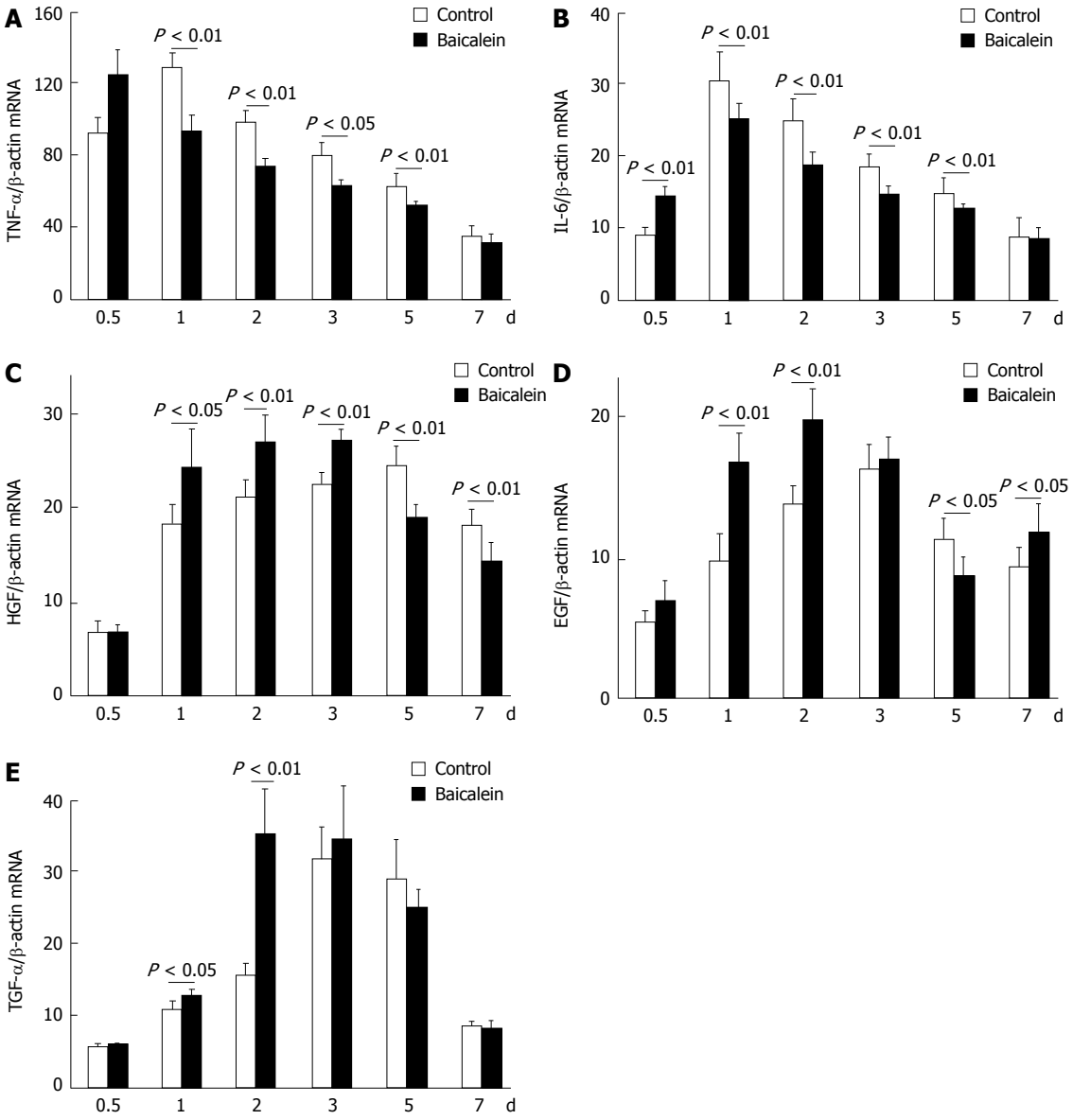

Figure 5 The microRNA levels of tumor necrosis factor-α, interleukin-6, transforming growth factor-α, hepatocyte growth factor and epidermal growth factor in liver of control and baicalein groups after carbon tetrachloride (1 mL/kg) treatment. Total RNA was isolated from liver tissue using TRIzol methods and quantified spectrophotometrically at 260 nm. The mRNA levels of tumor necrosis factor-α (*TNF-α*) (A), interleukin-6 (*IL-6*) (B), hepatocyte growth factor (*HGF*) (C), epidermal growth factor (*EGF*) (D) and transforming growth factor-α (*TGF-α*) (E) genes were quantified using reverse transcription polymerase chain reaction and normalized to *β-actin* housekeeping gene. Values represent mean ± SE ($n = 6$). $P < 0.05$, $P < 0.01$ between control and baicalein groups.

the production of several other proteins that are shared within the growth-factor-mediated pathway network. In previous studies, pretreatment with IL-6 before CCl_4 reduces acute CCl_4-mediated cell apoptosis, and accelerates regeneration in both wild-type and IL-6-/- livers[30]. The mechanism of IL-6 and TNF-α in protecting the liver against injury has not been fully clarified[31-33]. Previous studies showed that liver regeneration and hepatoprotection require the cytokine IL-6 immediately after liver injury[34,35]. But overexpression of IL-6 inhibits hepatocyte growth and causes liver injury[36,37]. In the present study, the expression of TNF-α and IL-6 in baicalein administrated mice reached a high level at day 0.5 and then was kept at a relatively lower level at days 1, 2, 3 and 5 compared with the control. We consider that the lower levels of TNF-α and IL-6 which are cell death mediators from days 1 to 5 may facilitate liver regeneration. In term of the mechanisms, we found that gene expression of *IL-6* and *TNF-α* in treated liver was enhanced in a similar pattern as the level of corresponding proteins, leading to the conclusion that baicalein could indeed alter the expression of certain cytokines to affect the liver damage process.

Another group of molecules we have investigated are growth factors such as HGF, TGF-α and EGF. They promote hepatic survival by stimulating liver regeneration and providing hepaprotection in various models of liver injury, such as toxic damage caused by CCl_4[38]. It has been proven that HGF, TGF-α and EGF are the main growth factors secreted after hepatic injury[39]. HGF is the most

potent mitogen for mature hepatocytes and acts as a hepatotropic factor. HGF level is increased markedly in mouse liver after various liver injuries such as hepatitis, ischemia, physical crush and partial hepatectomy. HGF acts as a trigger for liver regeneration and strongly enhances EGF expression. Previous studies indicated that the liver regenerative response is blocked if antibodies to HGF are administered at the same time as CCl_4 treatment[40]. HGF administration to rodents was confirmed to reduce the level of CCl_4-induced injury. HGF has been shown to regulate DNA synthesis partially through upregulation of other growth factors in hepatocytes *in vivo* and *in vitro*, which indicates that all of them are crucial for liver regeneration[41,42]. In our study, a significant increase of HGF, EGF and TNF-α expression occurred in livers from baicalein-treated groups during the proliferation phase (from days 1 to 3). Such expression reached a lower level in baicalein-treated mice at day 5 compared with the control, which indicated that the liver regeneration was terminated at an earlier phase.

In conclusion, we found baicalein from the Chinese herbal medicine possesses strong beneficial effects in a mouse model against acute liver injury caused by CCl_4. The expression of inflammatory cytokines IL-6 and TNF-α are markedly increased at the very early stage, which activate crucial signal transducers, including signal transducer and activator of transcription 3 and trigger certain signal cascades related to liver regeneration. During the middle stage, the expression level of such cytokines was significantly lowered to reduce inflammation cell apoptosis. The subsequent elevation of HGF, TGF-α and EGF may promote hepatic survival by stimulating hepatocyte regeneration. The protective effect of baicalein represents a clinical potential in the development of novel therapeutic agents for acute liver injury.

COMMENTS

Background

Baicalein is one of the bioactive compounds of *Scutellaria baicalensis Georgi* which has been shown to have anti-inflammatory, anti-bacteria and anti-hepatotoxic effects. However, the underlying mechanisms by which baicalein protects the liver from drug-induced injury still remain speculative.

Research frontiers

Previous investigations of liver diseases on the role of baicalein in selectively inducing apoptosis of cancer cells and inhibiting normal hepatocyte apoptosis, have prompted studies whether baicalein would increase the secretion of various inflammatory cytokines and facilitate regeneration of liver cells. This study assessed whether baicalein administration could prevent acute liver injury induced by carbon tetrachloride in mice and investigated the possible mechanism of its protective role.

Innovations and breakthroughs

The authors found that baicalein significantly elevated the serum level of tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 at the early phase, which indicated that baicalein could facilitate the initiating events in liver regeneration. This study supports the possibility that baicalein may be a therapeutic candidate for acute liver injury, and indicates that baicalein could accelerate liver regeneration by regulating the TNF-α and IL-6 mediated pathways.

Applications

All these results support the possibility of baicalein being a therapeutic candidate for acute liver injury, and indicate that baicalein accelerates liver regeneration by regulating the TNF-α and IL-6 mediated pathways.

Peer review

The authors concluded that baicalein could facilitate the initiating events in liver regeneration. The experiments were well done and the results were clearly shown. This study is well designed and performed, and is of great interest for its novelty and impact in the field.

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S- Editor Gou SX **L- Editor** Ma JY **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v18.i45.6614

World J Gastroenterol 2012 December 7; 18(45): 6614-6619
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Is proliferative colonic disease presentation changing?

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Supported by Grants from "Ateneo Federato" University "La Sapienza" Rome, Italy, Year 2009 - Protocol C26F098MZM

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Received: February 28, 2012 Revised: June 19, 2012
Accepted: August 4, 2012
Published online: December 7, 2012

Abstract

AIM: To compare the site, age and gender of cases of colorectal cancer (CRC) and polyps in a single referral center in Rome, Italy, during two periods.

METHODS: CRC data were collected from surgery/pathology registers, and polyp data from colonoscopy reports. Patients who met the criteria for familial adenomatous polyposis, hereditary non-polyposis colorectal cancer syndrome or inflammatory bowel disease were excluded from the study. Overlap of patients between the two groups (cancers and polyps) was carefully avoided. The χ^2 statistical test and a regression analysis were performed.

RESULTS: Data from a total of 768 patients (352 and 416 patients, respectively, in periods A and B) who underwent surgery for cancer were collected. During the same time periods, a total of 1693 polyps were analyzed from 978 patients with complete colonoscopies (428 polyps from 273 patients during period A and 1265 polyps from 705 patients during period B). A proximal shift in cancer occurred during the latter years for both sexes, but particularly in males. Proximal cancer increased > 3-fold in period B compared to period A in males [odds ratio (OR) 3.31, 95%CI: 2.00-5.47; $P < 0.0001$). A similar proximal shift was observed for polyps, particularly in males (OR 1.87, 95%CI: 1.23-2.87; $P < 0.0038$), but also in females (OR 1.62, 95%CI: 0.96-2.73; $P < 0.07$).

CONCLUSION: The prevalence of proximal proliferative colonic lesions seems to have increased over the last decade, particularly in males.

Key words: Colorectal cancer; Polyp; Location; Colonoscopy; Surgery

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Corleto VD, Pagnini C, Cattaruzza MS, Zykaj E, Di Giulio E, Margagnoni G, Pilozzi E, D'Ambra G, Lamazza A, Fiori E, Ferri M, Masoni L, Ziparo V, Annibale B, Delle Fave G. Is proliferative colonic disease presentation changing? *World J Gastroenterol* 2012; 18(45): 6614-6619 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i45/6614.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i45.6614

INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies worldwide, the etiology of CRC involves environmental and genetic factors. In most cases, the cancer develops according to the classic adenoma-carcinoma sequence[1], as supported by epidemiological, clinical-pathological and molecular genetic studies. Thus, early detection and removal of adenomatous polyps are essential for cancer prevention. In fact, the risk of developing CRC within six years of a polypectomy is reduced by 75%-90%. However, not all CRC cases are preceded by adenomatous polyps, and some cancers have been shown to develop directly from aberrant crypts or flat lesions[2]. These alternative carcinogenetic pathways are relatively more frequent on the right side of the colon, making the efficacy of preventative strategies very challenging. In this regard, one of the topics of particular interest in CRC is the possible change in site distribution observed in recent decades. In fact, recent data from different studies report a change in the site distribution of CRC (proximal shift) related to gender, race[3,4] and older age[5]. Many factors are potentially involved in this phenomenon, and most of them are not easily evaluated. However, the crucial issue is to establish if and how much of these changes are due to a real biological event or related to multiple diagnostic biases. Indeed, the question is not theoretical, but rather implies important decisions related to strategies for screening, surveying and treating millions of people worldwide, with health and economic implications. In fact, proximal colon cancer represents a great challenge for physicians, both due to the technical limitations of screening strategies in the detection of right-sided colon lesions and to the peculiar behavior of these tumors[6]. Data on CRC location have been reported from different sources, such as cancer registries, colonoscopy reports, retrospective clinical analyses or autoptic data[5,7-9]. All of these sources have biases that could potentially under- or over-estimate the specific issue of the cancer location. However, data concerning a possible increase over time of right-sided colon cancers have been reported recently in large population studies[10,11]. The possible changes in the location of polyps over time have been less investigated, but a possible proximal shift in these lesions has been described by some studies[12-14]. Few studies have addressed the possible "right shift" of CRC in the Italian population[15,16], and only two studies have analyzed the changing distribution of both CRC and polyps over tim[17,18], thus data are scarce and not conclusive.

The present study aims to address this issue retrospectively by analyzing records from a large set of patients, either operated on for CRC or diagnosed with colon polyps by colonoscopy, during two distinct periods of time at an Italian single referral center.

MATERIALS AND METHODS

We performed a retrospective, observational study of CRC and polyps at a single referral center ("Sapienza" University Hospital - Rome, Italy) for two periods of time: from 1989 to 1993 (period A) and from 2003 to 2007 (period B). The aim of the study was to compare the location of CRC and polyps and to study the differences in the age and gender distributions between the two periods.

The age and gender of the patients and the location, histology, morphology and dimensions of their lesions were recorded. For discrimination between the proximal and distal colon, the boundary was situated at the juncture of the splenic flexure, as was performed in previous studies[16,19].

Overlap of patients in the two groups (CRC and polyps) was carefully avoided. The study was approved by the institutional University review board; because this study was a retrospective analysis of an existing data set, written informed consent was not obtained from the participating subjects.

During the two periods, endoscopic examinations were performed using Olympus videocolonscopes (CF100I in period A, CFQ145I in period B).

Colorectal cancer data

CRC data were obtained from surgery registries, and the diagnoses were all confirmed by histological examination of surgical resections. Overall, 768 consecutive patients diagnosed with cancer who underwent surgery were analyzed. Of these, 352 were operated on from 1989-1993 (period A) and 416 from 2003-2007 (period B).

Polyp data

Polyp data were obtained from colonoscopies. Only complete colonoscopy examinations with adequate bowel preparation were considered. Subjects with uncompleted examinations or unsatisfactory cleansing were excluded, unless a second complete colonoscopy was performed within three months. Only patients with sporadic polyps were included, and patients who met the criteria for familial adenomatous polyposis, hereditary non-polyposis colorectal cancer syndrome or other polyposis syndromes, or who had been diagnosed with or suspected to have inflammatory bowel disease (ulcerative colitis or Crohn's disease), were excluded from the study.

Four senior gastroenterologists, each with more than 10 years of endoscopic experience, performed 4176 colonoscopies (1030 and 3146 for periods A and B, re-

Table 1 Characteristics of the 768 patients with colorectal cancer *n* (%)

	Period A (1989-1993)	Period B (2003-2007)	*P* value
	352 patients	416 patients	
Male	202 (57.4)	253 (60.8)	0.335
Female	150 (42.6)	163 (39.2)	
Age (yr)			
< 50	49 (13.9)	27 (6.5)	< 0.0001
50-59	70 (19.9)	55 (13.2)	
60-69	121 (34.4)	95 (22.8)	
70-79	90 (25.6)	180 (43.3)	
⩾ 80	22 (6.2)	59 (14.2)	

spectively). Polyps were detected in 27% and 23% of colonoscopies in periods A and B, respectively.

A total of 978 patients were analyzed, and 1693 polyps were found.

The data obtained from each polyp were included in the descriptive analysis. For patients with more than one polyp, the most advanced lesion, either in the proximal or in the distal segment of the colon, was taken into consideration in the multivariate analysis.

Statistical analysis

Proportions were calculated for the categorical data, and means and standard deviations were calculated for the quantitative data. χ^2 and *t* tests were used to assess the differences between periods A and B. Multivariate logistic regression was used to estimate the relative risk of finding a proximal CRC and polyp, adjusting for age, sex and the diagnosis period (A *vs* B) as independent variables. The limit of statistical significance for all tests was set at 0.05.

RESULTS

Colon cancer

As shown in Table 1, a higher percentage of cancers was recorded in men than in women, and there was no statistically significant difference between the periods. Patients were older in period B than in period A. In particular, there were fewer patients with CRCs in period B than in period A in all age groups less than 70 years.

From period A to B, proximal CRC incidence increased by an absolute 14.3% (from 18.2% to 32.5%, $P < 0.0001$). In particular, the increase was observed in the cecum and in the ascending colon (12.0% *vs* 28.4% in periods A and B, respectively), whereas in distal CRC cases, a consistent reduction was noted in the rectum, with a decrease from 54.5% to 31.5% in periods A and B, respectively ($P < 0.0001$) (Figure 1).

In the multivariate analysis, the risk of finding a proximal CRC, after adjusting for age and sex, showed a statistically significant interaction term between period B and gender. Thus, two regression equations were run. For men, the risk of developing a proximal cancer in period B was more than 3 times greater than that in period A, adjusting for age [odds ratio (OR) 3.31, 95%CI: 2.00-5.47; $P = 0.0001$], whereas for females, there was an

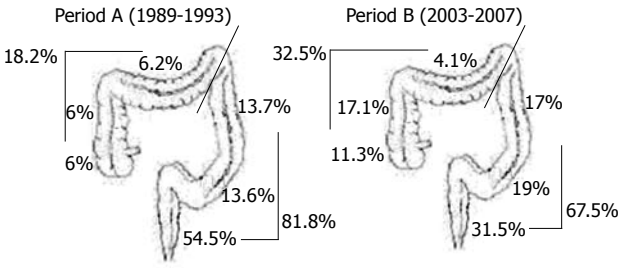

Figure 1 Relative distribution of colorectal cancer according to colon segment in periods A and B. The relative percentage of colorectal cancer cases are indicated next to the corresponding tract of the colon. Total proximal and distal colorectal cancer percentages, with the splenic flexure as the boundary, are also reported.

increased risk, but this increase was not statistically significant (OR 1.21, 95%CI: 0.72-2.04; $P = 0.4637$). There was no significant evidence of an effect of age in males (OR 1.34, 95%CI: 0.86-2.11; $P = 0.1999$) or in females (OR 1.23, 95%CI: 0.74-2.07; $P = 0.4272$).

Polyps

As shown in Table 2, polyps were more frequently found in males than in females, with no statistically significant difference between the periods ($P = 0.0892$). We evaluated 428 polyps from 273 patients in period A and 1265 polyps from 705 patients in period B. The mean number of polyps per patient increased from 1.6 in period A to 1.8 in period B ($P = 0.01$).

No univocal trend in age distribution between the two periods was observed. With regard to the percentage of patients with polyps in period A to period B, a decrease was observed for age groups < 50 years and 60-69 years, whereas an increase was observed for the age groups 50-59, 70-79 and ⩾ 80 years ($P < 0.0015$). A similar trend was observed in both males and females (data not shown).

From period A to B, the incidence of proximal polyps increased by an absolute 12.7% (from 22.8% to 35.5%, $P < 0.00005$). In analyzing the anatomical segments separately, an increase in incidence of proximal polyps was observed in the ascending colon (from 7.9% to 13.2%) and in the transverse colon (from 7.2% to 14.7%). In the distal colon, a reduction in polyps was observed in the descending colon (from 21.6% to 9.1%) and in the rectum (from 32% to 25.4%), whereas an increase was noted in the sigmoid colon (from 23.6% to 30%) ($P < 0.00005$) (Figure 2).

In the multivariate logistic regression analysis, after adjusting for age, a male's risk of developing a proximal polyp in period B was almost 90% greater than his risk in period A (OR 1.87, 95%CI: 1.23-2.87; $P = 0.004$), whereas for females, there was an increase of more than 60% in the risk, which was close to statistical significance (OR 1.62, 95%CI: 0.96-2.73; $P = 0.07$). When considering age groups stratified by greater or less than 70 years, no differences in proximal polyp detection was demonstrated for either gender.

The size and histopathological pattern of the polyps were also analyzed.

Table 2 Characteristics of the 978 patients with polyps *n* (%)

	Period A (1989-1993) 273 patients	Period B (2003-2007) 705 patients	*P* value
Male	178 (65.2)	418 (59.3)	0.0892
Female	95 (34.8)	287 (40.7)	
Age (yr)			
< 50	50 (19.2)	91 (12.9)	< 0.0015
50-59	44 (16.9)	154 (21.9)	
60-69	103 (39.5)	226 (32.1)	
70-79	57 (21.8)	185 (26.3)	
⩾ 80	7 (2.7)	48 (6.8)	
Total No. of polyps	428	1265	
No. of polyps, mean ± SD	1.6 ± 1	1.8 ± 1.3	0.0102
No. of polyps, median	1	1	
Range	1-6	1-14	
Dimensions			
< 5 mm	114 (26.6)	530 (41.9)	< 0.00005
5-9 mm	228 (53.3)	454 (35.9)	
10-19 mm	44 (10.3)	191 (15.1)	
20-29 mm	23 (5.4)	52 (4.1)	
30-39 mm	9 (2.1)	25 (2)	
40+ mm	10 (2.3)	13 (1)	
Histopathological pattern			
Hyperplastic	91 (33.6)	438 (38.7)	< 0.00005
Mild/moderate dispalsia[1]	151 (55.7)	597 (52.7)	
Severe dysplasia[2]	27 (10)	87 (7.7)	
Others[3]	2 (0.7)	10 (0.9)	

[1]Tubular, mixed or villous adenoma with mild or low grade dysplasia; [2]Tubular, mixed or villous adenoma with severe dysplasia or cancer in situ or serrated adenoma; [3]Lymphatic lump, hamartoma, leiomyoma, anal human papilloma virus.

There was a statistically significant increase in the percentages of micropolyps (< 5 mm) from 26.6% to 41.9% and from 10.3% to 15.1% for polyps of 10-19 mm in size, whereas the percentage of large polyps (40 mm) diminished from 2.3% to 1.0% in period B *vs* A ($P < 0.00005$).

Histopathology data were available for 63.3% and 89.5% of polyps in period A and B, respectively. No statistically significant differences in the overall number of hyperplastic polyps and adenomas with mild/moderate and severe dysplasia were observed between periods A and B. Nonetheless, when the histopathological pattern was analyzed according to polyp location, from period A to B, adenomas with mild/moderate dysplasia in the proximal colon increased significantly from 21.8% to 41.2% ($P < 0.001$), whereas adenomas with severe dysplasia decreased from 37% to 23%, which was not statistically significant.

DISCUSSION

In recent decades, screening strategies for early diagnosis and/or prevention of CRC have been consistently implemented. Nonetheless, colon malignancies still remain the third most common cancer and an important cause of death in Western countries[20]. Thus, many efforts have been made in order to improve the efficacy of screening strategies, which often differ even regionally in the same country. Colonoscopy is considered the "gold standard" for the diagnosis and removal of pre-malignant colon

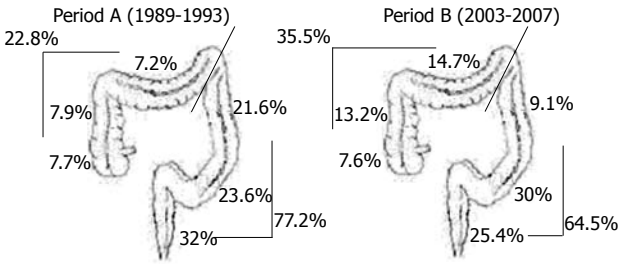

Figure 2 Relative distribution of polyps according to colon segment in periods A and B. Relative percentage of polyp detection are indicated next to the corresponding tract of the colon. Total proximal and distal colonic polyp percentages, with the splenic flexure as the boundary, are also reported.

lesions, even though a careful risk stratification strategy is required in order to optimize resources for screening purposes. In this setting, the presumed right-side increase in pre-malignant lesions and CRC may represent a further stimulus to perform high-quality endoscopic examination of the right side of the colon, which is often difficult to explore carefully (especially the cecum)[6]. Moreover, even though in the last year colonoscopies have increased in number and quality, it has been demonstrated that a relatively high proportion of cases of CRC may develop without macroscopic evidence of pre-malignant lesions, introducing further challenges to prevention strategies. Despite the consistent number of studies that analyze differences in the location of colon CRC and polyps, data are still not univocal and indeed remain difficult to interpret. As already mentioned, results are often difficult to compare due to the different sources from which the data are collected. Another important reason concerns the length of the observation, which varies from a few years to decades according to different studies.

This study retrospectively evaluated the differences in the site distribution of CRC and polyps between two 5-year periods over a period of 10 years, analyzing data from surgical registries and from endoscopic reports in a single referral center. The relatively short interval time between the two periods (10 years) could have, at least in part, influenced the observed differences, which may be more striking with a wider interval time.

Bearing in mind the aforementioned limitations for data interpretation, many recent large studies have reported a trend for "proximalization" of CRC in different geographic areas[10,15,21,22]. Conversely, other studies have questioned the possible "right shift" in CRC location[23,24] or have observed the phenomenon only in specific subgroups[3,25,26]. Moreover, some other authors have explained that the putative increase in proximal CRCs is mainly consequent to the decrease in rectal cancer cases[8,16]. In this study, we confirmed the proximal shift in CRC over time and observed a 3-fold increase in the risk of finding proximal cancer in males in period B *vs* period A. In line with previous findings in Italian populations[16], the single anatomical segments analysis emphasized that the relative increase in proximal CRC cases over time was partly due to a reduction in the number of rectal cancer cases (54.5% *vs*

31.5% in periods A and B, respectively). In fact, excluding rectal cancers, the trend for a proximal shift in CRCs over time, although maintained, showed less of a difference (data not shown). This relatively small, but homogeneous study from a single referral center, confirmed the trend of a proximal shift in CRC location during recent years, and is thus further confirmation of the phenomenon previously described in large cohorts of patients from different areas.

Published data on polyp prevalence are scarce and less consistent than are data for CRC, and the related studies mainly concern advanced adenomas[27]. However, some studies have suggested a proximal shift in those lesions over time[12-14,17,18]. In this study, we observed a proximal shift in polyps between periods A and B, albeit less consistent than that observed for CRC. As already observed for CRC, the proximalization of lesions was more evident in males (90% increase in period B *vs* A). The increase in total polyps, and in particular in proximal lesions, refers mainly to micropolyps and low-grade dysplastic polyps, that could be partially explained by the increase in colonoscopies for cancer prevention in period B *vs* period A, and to the "see and sampling" strategy that has become more popular in recent years. Notably, the present data on polyp dimensions and histopathological patterns need to be interpreted with caution, both due to the high rate of missing histological data [157 (36.7%) and 133 (10.5%) polyps with missing histological reports in periods A and B, respectively] and due to the fact that the two variables (size and histology) are not independent.

Besides possible biological explanations of increased proliferative right-sided colon lesions over time, many confounding factors related to the global technical and behavioral medical changes throughout the years could have partially contributed to this location shift. With regard to the latter, the most important consideration concerns the impact of increased sensibilization for CRC prevention in the last decade that could have potentially influenced either CRC or polyp presentation in our population during period B. In fact, the older age of CRC patients in that period could be at least partially due to the preventive effect of the screening approach, and the same "proximal shift" could be an effect of better prevention of distal lesions (which are more easily detected by screening methods such as sigmoidoscopy). Considering polyps, the modifications of colonoscopy indications, particularly due to an increased trend to cancer prevention, may have influenced the different findings in the two periods, even though only a slight decrease in the proportion of colonoscopies with polyp detection was found between the two periods (23% in period B *vs* 27% in period A). Regarding technical progress, the right-sided CRC increase could be a result of the recent different surgical treatment options for right-sided CRC, in particular the laparoscopic approach, that in many centers has made surgery much more possible in elderly patients compared to previous years. This detail is particularly true considering that for CRC evaluation, we included exclusively surgical registry data without considering the surgical approach. Moreover, procedural improvements (i.e., standardization of retraction time) and the amelioration of bowel cleansing could have potentially influenced the observed difference in polyp detection between the two time periods. Nonetheless, no substantial improvements in technical equipment occurred between the two periods, since high definition endoscopes were not available in both periods.

Nonetheless, even if the precise amount and specific causes of the right shift in pre-malignant and malignant colon lesions remain to be established, the present retrospective analysis appears to confirm, albeit with some limitations and possible confounding factors, a trend of an increase in such lesions over time. As a consequence, endoscopists and clinicians in daily clinical practice, as well as future strategies for screening campaigns, should take into account the possible increase in proximal colonic proliferative disorders. In this regard, the whole colon should be considered as a potential target for neoplastic changes, and partial colon examinations should be avoided or limited to particular conditions. Novel endoscopic instruments with higher resolution power could result in an improvement *per se* in the detection of colonic lesions. However, besides the technical devices, better bowel preparation (cecum cleaning), the constant improvement of endoscopists' skills, and a standardized technical endoscopic approach[28] are all fundamental basic tools that can improve the endoscopic examination quality in order to obtain a more accurate observation of the whole colon.

COMMENTS

Background

Proximalization of colorectal cancer during the last decades has been variously reported. Data for the right shift in polyps are scant and controversial.

Research frontiers

Data from large cohorts of patients followed for decades and relatively short studies suggest a change in colonic proliferative disease during recent years, with an increase in right-sided lesions.

Innovations and breakthroughs

The data show a proximalization of proliferative colonic lesions (cancers and polyps), in a single referral center, in two different periods of time with a ten-year interval between these periods.

Applications

These results should be interpreted with caution, due to many possible unavoidable biases that may interfere when undertaking this type of study, either for different endoscopical/surgical approaches or for biological factors. However, the authors suggest that this phenomenon that may have important implications either for improvement of endoscopic accuracy or for screening programs.

Peer review

The study involves a single institution but covers 2 separate 5 year periods 10 years apart. The authors compared data regarding the anatomic location of both colorectal cancers and colonic polyps found by colonoscopy during each period. The data suggest that proximal proliferative lesions have increased in prevalence, particularly in males. That proximal colonic premalignant and malignant conditions have increased in prevalence over the last 2 decades has also been shown in other studies. In order to be sure that comparisons between 2 separate periods are valid, even within a single institution, the procedures in question should be as identical as possible, allowing for changes in technology, operator experience, *etc*.

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Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v18.i45.6620

World J Gastroenterol 2012 December 7; 18(45): 6620-6627
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Value of adipokines in predicting the severity of acute pancreatitis: Comprehensive review

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Received: July 10, 2012 Revised: September 18, 2012
Accepted: September 22, 2012
Published online: December 7, 2012

Abstract

AIM: To analyze the prognostic value of adipokines in predicting the course, complications and fatal outcome of acute pancreatitis (AP).

METHODS: We performed the search of PubMed database and the systemic analysis of the literature for both experimental and human studies on prognostic value of adipokines in AP for period 2002-2012. Only the papers that described the use of adipokines for prediction of severity and/or complications of AP were selected for further analysis. Each article had to contain information about the levels of measured adipokines, diagnosis and verification of AP, to specify presence of pancreatic necrosis, organ dysfunction and/or mortality rates. From the very beginning, study was carried out adhering to the PRISMA checklist and flowchart for systemic reviews. To assess quality of all included human studies, the Quality Assessment of Diagnostic Accuracy Studies tool was used. Because of the high heterogeneity between the studies, it was decided to refrain from the statistical processing or meta-analysis of the available data.

RESULTS: Nine human and three experimental studies were included into review. In experimental studies significant differences between leptin concentrations at 24 and 48 h in control, acute edematous and acute necrotizing pancreatitis groups were found (P = 0.027 and P < 0.001). In human studies significant differences between leptin and resitin concentrations in control and acute pancreatitis groups were found. 1-3 d serum adiponectin threshold of 4.5 μg/mL correctly classified the severity of 81% of patients with AP. This threshold yielded a sensitivity of 70%, specificity 85%, positive predictive value 64%, negative predictive value88% (area under curve 0.75). Resistin and visfatin concentrations differ significantly between mild and severe acute pancreatitis groups, they correlate with severity of disease, need for interventions and outcome. Both adipokines are good markers for parapancreatic necrosis and the cut-off values of 11.9 ng/mL and 1.8 ng/mL respectively predict the high ranges of radiological scores. However, the review revealed that all nine human studies with adipokines are very different in terms of methodology and objectives, so it is difficult to generalize their results. It seems that concentrations of the leptin and resistin increases significantly in patients with acute pancreatitis compared with controls. Serum levels of adiponectin, visfatin and especially resitin (positive correlation with Acute Physiology and Chronic

Health Evaluation Ⅱ, Ranson and C-reactive protein) are significantly different in mild acute pancreatitis and severe acute pancreatitis patients, so, they can serve as a markers for the disease severity prediction. Resistin and visfatin can also be used for pancreatic and parapancreatic necrosis prediction, interventions needs and possible, outcome.

CONCLUSION: High levels of adipokines could allow for prediction of a severe disease course and outcome even in small pancreatic lesions on computed tomography scans.

Key words: Adipokines; Acute; Pancreatitis; Severity; Prediction

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INTRODUCTION

Acute pancreatitis (AP) is a common disease with a wide spectrum of severity. Its incidence is about 30-113 cases per 100 000 individuals, with the overall mortality rate of 10%-15%[1-4]. Most episodes of AP are mild and self-limiting, but up to 10%-20% of patients develop severe AP with mortality ranging from 29% to 43%[5,6]. Over past two decades mortality rate in early phase of AP associated with the systemic inflammatory response syndrome decreased significantly, but mortality in the late phase remains high. It is believed that the cause of death in late phase is predominantly linked to the development of infected necrosis and septic complications[7], thus resulting in multiple organ failure and severe sepsis[8,9]. Because of the systemic complications and high mortality, a considerable interest in the early prediction of the disease course and severity remains.

Pancreatic enzyme levels poorly correlate with the severity of AP, thus prognosis is commonly based on clinical scores. The first disease specific prognostic score was proposed by Ranson in 1974, which later was complemented by a number of pancreatitis specific and organ failure scores, including Glasgow/Imrie (1984), Acute Physiology and Chronic Health Evaluation (APACHE) Ⅱ (1985), Multiple Organ Dysfunction Score (1995), Sequential Organ Failure Assessment (1998), Pancreatitis Outcome Prediction (2007), Bedside Index of Severity in Acute Pancreatitis (2009), and many others. Although, the accuracy of such scores is high enough (Table 1)[10-14], all of them are multifactorial and rather uncomfortable for everyday use, so a great attention is still given for seeking a single prognostic marker. The most widely explored and described single predictor is C-reactive protein (CRP), which remains very useful, because it is accurate, cheap, and widely available. However, its concentration reaches a peak on third day of the disease, so it has a greatest prognostic value approximately 48 h after the onset of the symptoms. Optimal cut-off value recommended by almost all societies for disease course prediction is 150 mg/L. CRP has sensitivity of 80%, specificity of 84% with area under curve (AUC) 0.84 in predicting severity of the disease 48 h after admission with a cut-off of 150 mg/L[15]. There is little data on the value of CRP on prediction of development of pancreatic necrosis. Some studies demonstrated that a cut-off of as low as 71 mg/L is sufficient to predict development of clinically significant (volume > 30%) necrosis with a sensitivity of 78.79%, specificity of 71.43% and AUC 0.766[12].

The main problem remains, that neither prognostic scores nor single predictors can't accurately predict the disease course and severity, development of pancreatic or peripancreatic necrosis, and outcomes during the first hours or even days of hospitalization. Therefore, there is a great stimulus for seeking new accurate and easy to use predictors. Perhaps, the least studied group of predictors in AP is adipokines, including adiponectin, leptin, resistin and visfatin.

Adiponectin is being produced exclusively in adipocites and plays an important role in the inhibition of the inflammatory response[16,17]. Adiponectin depresses nuclear factor kappa B signaling in endothelial cells and adipocytes, induces the anti-inflammatory cytokine interleukin (IL)-10 and IL-1 receptor antagonist in leukocytes[18-20].

Leptin is an adipocyte-derived hormone that acts centrally in the hypothalamus to regulate body waste and peripherial energy expenditure[21]. The presence of leptin and expression of its receptors have been detected in other tissues, also in pancreas[22]. This suggests, that leptin may modulate pancreatic function and inflammatory response in pancreatitis.

Resistin and visfatin are the adipohormones, produced by neutrophils, macrophages, bone marrow and WAT[23,24]. They can induce the synthesis of pro-inflammatory cytokines, such as IL-6, IL-1β, tumor necrosis factor alpha, that is why their role in inflammatory response has been suggested[24-27].

It is now widely accepted, that white adipose tissue is an active endocrine organ, which is also involved in pathogenesis of AP. Peripancreatic fat cells necrosis might cause a massive release of cytokines (IL-1, IL-6, tumor necrosis factor) and adipokines, that possibly cause multiorgan dysfunction and whole body metabolic changes. It is hypothesized that the extent of peripancreatic fat-cell necrosis determines the severity of pancreatitis, and an early increase of adipocyte-specific marker proteins might serve as predictor of the clinical course[28].

Table 1 Pancreatitis prediction scores

Score	Sensitivity (%)			Specificity (%)			AUC		
	Course	Necrosis	Mortality	Course	Necrosis	Mortality	Course	Necrosis	Mortality
Ranson, 1974	84	77	100	90	88	77	0.94	0.85	0.95
Glasgow/Imrie, 1984	70	82	89	83	73	70	0.84	0.82	0.80
APACHE Ⅱ, 1985	70	63	100	72	69	66	0.78	0.72	0.90
MODS, 1995	73	69	89	81	74	90	0.84	0.78	0.93
SOFA, 1996/1998	76		87	69		90	0.81		0.93
POP, 2007	83	51	78	71	95	86	0.86	0.71	0.89
BISAP, 2009	38	33	57	92	91	88	0.81	0.78	0.82

APACHE: Acute Physiology and Chronic Health Evaluation; MODS: Multiple Organ Dysfunction Score; SOFA: Sequential Organ Failure Assessment; POP: Pancreatitis Outcome Prediction; BISAP: Bedside Index of Severity in Acute Pancreatitis; AUC: Area under curve.

Table 2 Reviewer judgments of methodological quality of included human studies according to the Quality Assessment of Diagnostic Accuracy Studies tool

	Konturek *et al*[30]	Leśniowski *et al*[33]	Duarte-Rojo *et al*[34]	Tukiainen *et al*[35]	Sharma *et al*[36]	Schäffler *et al*[37]	Schäffler *et al*[38]	Schäffler *et al*[39]	Daniel *et al*[40]
Patients spectrum	No	No	Yes	Yes	No	Yes	Yes	Yes	Yes
Selection criteria	Unclear	No	No	No	Yes	No	No	No	Yes
Reference standart	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes
Period between IT and RS	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Verification	Unclear	Yes	No	Yes	Yes	No	No	No	Yes
Same RS	No	Yes	No	No	Yes	No	No	No	Yes
RS independence on IT	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
IT replication	Yes	No	No	No	No	No	Yes	Yes	Yes
RS replication	No	No	No	No	No	No	No	No	No
IT interpretation	No	No	No	No	No	No	No	No	No
RS interpretation	Yes	No	No	No	No	No	No	No	No
Data in practice	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Report	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Withdrawals	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Total	8 yes	7 yes	6 yes	8 yes	9 yes	7 yes	8 yes	8 yes	11 yes

IT: Index test; RS: Reference standart.

The aim of this study is to analyze and review the available information about the prognostic value of adipokines in predicting the course of AP, development of pancreatic and peripancreatic necrosis, infectious complications, need for interventional treatment, and fatal outcome. The main objective of the study is to compare the prognostic value of adipokines with already well established single predictors and multifactorial scores in the clinical context.

MATERIALS AND METHODS

We performed the search of PubMed database (service of the United States National Library of Medicine that includes citations from MEDLINE and other life science journals for biomedical articles) and the systemic analysis of the literature for both experimental and human studies on prognostic value of adipokines in AP for period 2002-2012. Keywords (keywords and textwords) for the search were adipokines, adipocitokines, visfatin, resistin, adiponectin, leptin, acute pancreatitis, pancreatic necrosis, peripancreatic necrosis. Further we searched the references of identified articles to find additional sources of information. Only articles in English language were included in the analysis. Dual publications were excluded. All identified papers (title, abstract and subsequently full text) were independently evaluated by two investigators. Only the papers that described the use of adipokines for prediction of severity and/or complications of AP were selected for further analysis. To be included in the systematic review, each article had to contain information about the levels of measured adipokines, diagnosis and verification of AP, to specify presence of pancreatic necrosis, organ dysfunction and/or mortality rates. All disagreements were resolved by discussion with other two investigators. From the very beginning study was carried out adhering to the PRISMA checklist and flowchart for systemic reviews.

To assess quality of all included human studies the Quality Assessment of Diagnostic Accuracy Studies tool was used[29]. Quality assessment was performed independently by three researches and all disagreements were resolved by review and discussion with the fourth investigator. The result of the human studies quality assessment is shown in Table 2. Based on the judges' evaluation 8 of 9 studies got seven or more "yes", so the overall quality of included studies was good. However, all studies were very different. Four of them analyzed only one adipo-

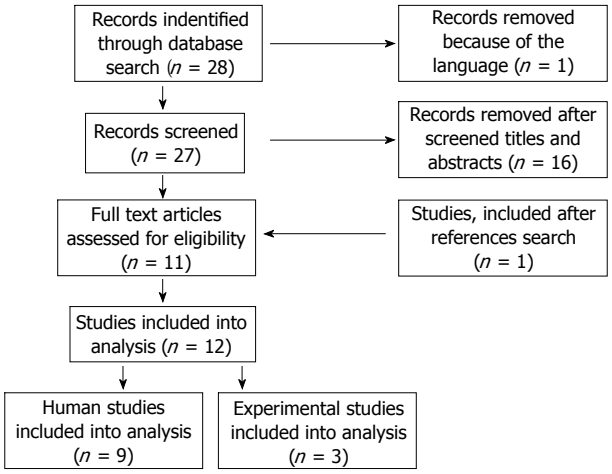

Figure 1 Selection of the studies for systematic review (PRISMA flowchart). Records identified through database search, *n* = 28.

kine, two adipokines were analyzed in three studies, and the remaining two studies analyzed three adipokines. In two studies adipokines concentration was measured only in control and AP groups, without distinction of mild and severe acute pancreatitis.

Statistical analysis

Because of the high heterogeneity between the studies, lack of the uniform diagnostic criteria and high variation of the assessed adipokines profile it was decided to refrain from the statistical processing or meta-analysis of the available data.

RESULTS

Through database search 28 records were identified. After screening the titles and abstracts, 16 records were removed, because adipokines were not used for prediction of the disease course. One record was removed because of the language. In reference search one additional study was found. So, nine human and three experimental studies were further analyzed (Figure 1).

All three experimental studies were performed on rats. The only one adipokine leptin was analyzed. In all studies significant differences between leptin concentrations in control and acute pancreatitis groups was found[30-32], one study analyzed leptin concentrations in control, acute edematous pancreatitis (AEP) and acute necrotizing pancreatitis (ANP). Significant difference at 12 h was found between controls and ANP group. At 24 and 48 h significant difference was found between controls and both AEP and ANP groups[32] (Table 3).

All nine human studies (Table 4) with adipokines are very different in terms of methodology and objectives, so it is difficult to generalize their results. It seems that concentrations of the leptin and resistin increases significantly in patients with AP compared with controls. Serum levels of adiponectin, visfatin and especially resitin (positive correlation with APACHE Ⅱ, Ranson and CRP) are significantly different in severe acute pancreatitis (SAP), mild acute pancreatitis (MAP) patients, so, they can serve as a markers for the disease severity prediction. Resistin and visfatin can also be used for pancreatic and parapancreatic necrosis prediction, interventions needs and possible, outcome.

Table 3 Summary of the experimental studies on the prognostic value of adipokines in rats

Study	Groups	*n*	Leptin, ng/mL	*P* value
Konturek *et al*[30]	AP	6-8	7.5 (4.3-18.4)	$P < 0.01$[1]
	Controls	6-8	2.1 (1.0-11.8)	
Yavuz *et al*[31]	AP	10	1.92 ± 0.1	
	CP	10	1.86 ± 0.13	
	Controls	10	0.78 ± 0.12	$P < 0.001$
Kerem *et al*[32]	AEP	30		$P < 0.001$[2]
	ANP	30		
	Controls	30		$P = 0.027$ and $P < 0.001$

AP: Acute pancreatitis; CP: Chronic pancreatitis; AEP: Acute edematous pancreatitis; ANP: Acute necrotizing pancreatitis; CIP: Caerulein-induced pancreatitis. [1]The induction of CIP resulted in a significant increase of plasma levels of leptin; [2]At 12 h leptin levels in ANP was higher than in controls, at 24 and 48 h leptin levels in AEP and ANP were higher than in controls.

DISCUSSION

Human studies (Table 4) began in 2002, when Konturek *et al*[30] found, that median plasma leptin levels in AP were significantly increased as compared with controls. In 2007, Leśniowski *et al*[33] found a significant differences between resistin concentrations in AP and control groups.

In study of Duarte-Rojo *et al*[34] there was no significant independent association between leptin serum levels and severity of AP or fatal outcome. The similar results are published from Tukiainen *et al*[35]: on admission plasma leptin levels do not correlate with AP severity. This study also did not confirm correlation between adiponectin levels and severity of AP. Despite of this, in 2009, Sharma *et al*[36] has shown, that 1-3 d serum adiponectin threshold of 4.5 μg/mL correctly classified the severity of 81% of patients with AP. This threshold yielded a sensitivity of 70%, specificity 85%, positive predictive value 64%, negative predictive value 88% (AUC 75%).

Promising results are published from Schäffler *et al*[37-39] group. They began their trial in 2006 and finished in 2011 with 41 SAP and 9 MAP patients. This study has shown, that resistin and visfatin concentrations has significant differences between MAP and SAP groups, they correlate with severity of disease, need for interventions and outcome. Both adipokines are good markers for parapancreatic necrosis and the cut-off values of 11.9 ng/mL and 1.8 ng/mL respectively allow to predict the high ranges of radiological scores. These results are consistent with Daniel *et al*[40] study in 2010, which demonstrates, that resistin and visfatin may be possibly used for AP prognosis and disease monitoring.

Although Schäffler group provides some cut-off values of adipokines, which are associated with high radiological

Table 4 Summary of the human studies on the prognostic value of adipokines

Study	Patients and methods	Results	Conclusions
Konturek *et al*[30]	Prospective observational study (n = 45) Diagnosis of AP based on Atlanta criteria Adipokines studied: leptin Adipokines evaluated between 48-72 h of illness onetime AP (n = 15) *vs* controls (n = 30)	Leptin: AP/controls- 7.5 (4.3-18.4) ng/mL/2.1 (1.0-11.8) ng/mL	Median plasma leptin levels in AP were significantly increased as compared with controls
Duarte-Rojo *et al*[34]	Prospective observational study (n = 52) Diagnosis of AP based on typical clinical manifestations with at least a 3-fold increase of serum amylase and/or lipase Whenever uncertainty about diagnosis existed, CT-scan was performed to confirm/rule out AP Severe AP was considered when patients developed one or more local or systemic complications according to the Atlanta classification of AP Adipokines studied: leptin Adipokines evaluated onetime during the 1 d of hospital stay MAP (n = 38) *vs* SAP (n = 14)	There was no statistically significant association between leptin serum levels and severity of AP There was no difference in leptin measurements between patients favorable and fatal outcomes (P = 0.34) Time of evolution from onset of pain did not alter leptin values There was a positive correlation of BMI and leptin (r = 0.476, P < 0.001) in the whole group Predicted severity by modified Ranson's criteria correlated with Atlanta criteria (r = 0.414, P = 0.002); however, it did not correlate with leptin levels	Results do not support human leptin as a major pro-inflammatory signal involved in AP, nor as a protective and anti-inflammatory mediator It seems neither to be the link between obesity and a higher rate of complications in AP; nor a prognostic marker
Tukiainen *et al*[35]	Prospective observational study (n = 24) AP and SAP defined by Atlanta criteria Adipokines studied: leptin, adiponectin Adipokines evaluated on admission, on days 2-4, and on days 5-7 MAP (n = 12) *vs* SAP (n = 12)	In patients with SAP highest value of CRP was 349 mg/L (284-476 mg/L), with MAP 119 mg/L (11-367 mg/L) Leptin on admission SAP/MAP [6.1 (1.6-72.9) ng/L]/[9.0(2.5-36 .5) ng/L], (P > 0.05); on days 2-4, 7.7 (1.6-13.9) ng/L/3.8(1.6-12.9) ng/L, (P > 0.05) Adiponectin on admission SAP/MAP, [5642 (1201-19 400) ng/L]/[6314 (1980-24 340) ng/L], (P > 0.05)	Plasma levels of adiponectin and leptin do not correlate with AP severity on admission and during the first week of the disease
Schäffler *et al*[37]	Pilot prospective observational study (n = 23) Diagnosis of AP was based on clinical, laboratory and radiological findings during CT and/or ultrasound examination Adipokines studied: leptin, adiponectin, resistin Adipokines evaluated daily for 10 d after admission SAP (n = 20) *vs* MAP (n = 3) and patients with high points *vs* low points on radiological scores	Balthazar score: 4 (1-5), Schroeder score: 5 (1-7), Necrosis score: 2(1-4) Ranson: 3 (0-7), Apache II: 12 (4-37) Resistin has a significant positive correlation with Ranson score (r= 0.6, P = 0.002) and with Apache II score (r = 0.5, P = 0.019) Resistin: intervention group/no intervention, 32.4 ± 10.7 ng/L/15.8 ± 5.1 ng/L, P = 0.026 Leptin and relative changes in leptin values were positively and significantly correlated with CRP levels (r = 0.6, P = 0.007 and P = 0.003, respectively) Resistin cut-off value of > 9.2 ng/mL (10 d mean value) can provide a PPV of 91.9% in predicting Schroder score of > 3 (specificity 85%, sensitivity 75%, AUC 0.9, P < 0.0001) Leptin cut-off value of 15.0 ng/mL can provide a PPV of 88% in predicting Schroder score of > 3 (specificity 85%, sensitivity 50%, AUC 0.72, P < 0.0001) Day 1 resistin proved to predict a Schroder score > 3 with a PPV of 93.3%, cut-off 6.95 ng/mL, specificity 87.5%, sensitivity 93.3%; AUC 0.9, P = 0.002)	Serum adipokines might be the new useful early markers of disease severity in AP
Leśniowski *et al*[33]	Prospective observational study (n = 79) All AP was classified as grade B according to Balthazar CT score Adipokines studied: adiponectin, resistin Adipokines evaluated onetime during the first day of hospitalization AP (n = 39) *vs* controls (n = 40)	Resistin: AP/controls, 8.38 ± 4.87 ng/mL/3.58 ± 1.51 ng/mL, P < 0.05 Adiponectin: AP/controls, 119.38 ± 61.75 ng/mL/133.77 ± 55.38 ng/mL, P > 0.05 CRP: AP/controls, 23.21 ± 8.75 ng/mL/3.95 ± 1.06 mg/L, P < 0.01 Weak positive correlation between serum resistin and CRP was observed (r = 0.57, P < 0.05) No correlation between selected adipocytokines and BMI was noticed	Serum concentrations of resistin may possibly represent the useful early marker of inflammatory response in AP
Sharma *et al*[36]	Prospective observational study (n = 60) Diagnosis of AP based on Atlanta criteria SAP was defined as the presence of cardiovascular, pulmonary, and/or renal system dysfunction during the initial hospital admission during for at least 48 h Adipokines studied: adiponectin Adipokines evaluated on admission and subsequently up to 30th hospital day MAP (n = 27) *vs* SAP (n = 33)	Serum adiponectin levels from days 1 to 3 were significantly lower for patients with SAP [median 3.74 (0.83-8.92) μg/L] than those with MAP [6.58 (1.31-15.37) μg/L], P = 0.02 Serum adiponectin levels from days 4 to 7 were lower for patients with SAP [median 4.53 (0.94-18.2) μg/L] than those with MAP [8.06 (2.11-17.72) μg/L], P = 0.01 1-3 d serum adiponectin threshold of 4.5 μg/mL correctly classified the severity of 81% of patients with AP This threshold yielded a sensitivity of 70%, specificity 85%, PPV 64%, NPV 88%, AUC 0.75	Serum adiponectin levels are significantly lower in patients with SAP than those with MAP and could serve as inverse marker of systemic inflammatory response to pancreatic injury

Daniel *et al*[40]	Prospective observational study (n = 62) Diagnosis of AP was based on at least threefold elevated serum amylase level, as well as ultrasonography and CT In all cases AP was classified as C according to Balthazar's CT score and as severe according to Ranson's criteria (3 points) Adipokines studied: resistin Adipokines evaluated on 1, 2, 3 and 5 d of hospitalization SAP (n = 32) *vs* controls (n = 30)	On first day of observation , the median serum CRP level was 51.9 ± 46.1 mg/L, significantly higher than in control group (3.44 ± 3.04 mg/L, P = 0.01), and further increased at third day of hospitalization (102.6 ± 55.1 mg/L, P < 0.05), slightly decreasing on fifth day of hospitalization (78 ± 47.7 mg/L) The values observed at third and fifth day of hospitalization were significantly higher than in the control group (P < 0.001) One day of admission and third day of the hospitalization the mean serum resistin concentration was 12.9 ± 6.38 ng/mL and 17.4 ± 4.23 ng/mL, respectively Both values were significantly higher than in the control group (4.06 ± 2.63 ng/mL, P < 0.05) At fifth day of hospitalization serum resistin concentration increase further to 25.8 ± 8.14 ng/mL, which was significantly higher than at first and third day (P < 0.05) of hospital stay Significant correlation between CRP and resistin (r = 0.43, P < 0.05) during the hospital stay was found	Resistin may be useful early marker in edematous form of AP
Schäffler *et al*[38,39]	Prospective observational study (n = 50) Diagnosis of AP was based on clinical, laboratory and radiological findings during CT and/or ultrasound examination All patients were divided into three groups: first - with higher radiological score's points, second - with lower radiological score's points and third - no CT scan (mild pancreatitis) Adipokines studied: leptin, adiponectin, resistin, visfatin Adipokines were measured daily from admission till 10 d of hospital stay SAP (n = 41) *vs* MAP (n = 9) and patients with high points *vs* low points on radiological scores	Balthazar score: 4.0 (1-5), Schroeder score: 4.5 (1-7), Necrosis score: 1.5 (1-4), Ranson: 3 (0-8), Apache II: 12 (0-45) Admission resistin levels has positive an significant correlation with Apache II score (r = 6, P < 0.001) and with Ranson score (r = 0.4, P = 0.013) Admission resistin cut-off value of > 11.9 ng/mL can provide a PPV of 89% in predicting Schroeder score of > 3 (specificity 80%, sensitivity 70%, AUC 0.8, P < 0.002) Admission resistin cut-off value of > 11.9 ng/mL can serve as a positive predictor of a Balthazar score > 3 and Necrosis score > 2 Admission visfatin cut-off value of > 1.8 ng/mL can provide a PPV of 93.3% in predicting Schroeder score of > 3 (specificity 81.8%, sensitivity 93.3%, AUC 0.89, P < 0.001, likehood ratio 5.1, post-test probability 93.0%) Admission visfatin concentration can also predict Necrosis score > 2 (PPV 48.3, specificity 40.0%, sensitivity 93.8%, AUC 0.77, P < 0.004, likehood ratio 1.5, post-test probability 70.0%) and Balthazar score > 3 (PPV 79.3, specificity 57.1%, sensitivity 88.9%, AUC 0.74, P < 0.011, likehood ratio 2.1, post-test probability 55.0%)	Resistin and visfatin levels are highly elevated in patients with SAP when compared to patients with MAP Both adipokines levels are positively correlated with clinical severity, clinical end points and needs for interventions A single measurement of serum resistin or visfatin on the day of admission is a highly significant and positive predictive marker in predicting peripancreatic necrosis

AP: Acute pancreatitis; SAP: Severe acute pancreatitis; MAP: Mild acute pancreatitis; BMI: Body mass index; CRP: C-reactive protein; AUC: Area under curve; PPV: Positive predictive value; NPV: Negative predictive value; CT: Computed tomography.

scores ranges, there is no precise cut-off values in order to predict disease severity on admission, development of pancreatic and parapancreatic necrosis, infectious complications, need for interventional treatment and fatal outcome. Therefore it is difficult to compare the prognostic value of adipokines with other prognostic systems.

It is clear, that obesity complicates the course of acute pancreatitis and it is associated with higher incidence of local complications, organ failure and increased mortality risk[41,42]. Adipose tissue doesn't accumulate contrast, making it difficult to evaluate it's necrosis on computed tomography (CT) scans. Thus, adipokines could be a useful markers for adipose tissue necrosis. High levels of adipokines could allow for prediction of a severe disease course and outcome even in small pancreatic lesions on CT scans, but further research is needed. Therefore, it is appropriate to initiate a multicenter study, with a sufficient number of AP patients and controls. All patients must be evaluated with the same clinical score, adipokines should be investigated all at once and CT scans should be standardized in time. We believe, that such a study could provide a more definitive answer about the value of the adipokines in predicting the course and the outcomes of AP in a clinical setting.

COMMENTS

Background

Acute pancreatitis (AP) is a common disease with a wide spectrum of severity. The main problem remains, that neither prognostic scores nor single predictors can't accurately predict the disease course and severity, development of pancreatic or peripancreatic necrosis, and outcomes during the first hours or even days of hospitalization. Therefore, there is a great stimulus for seeking new accurate and easy to use predictors. Perhaps, the least studied group of predictors in AP is adipokines. The aim of this study was to analyze the prognostic value of adipokines in predicting the course, complications and fatal outcome of AP.

Research frontiers

It is now widely accepted, that white adipose tissue is an active endocrine organ, which is also involved in pathogenesis of AP. Peripancreatic fat cells necrosis might cause a massive release of and adipokines, that possibly cause multi-organ dysfunction and whole body metabolic changes. It is hypothesized that the extent of peripancreatic fat-cell necrosis determines the severity of pancreatitis, and an early increase of adipocyte-specific marker proteins might serve as predictor of the clinical course.

Innovations and breakthroughs

It seems that concentrations of the leptin and resistin increases significantly in patients with AP compared with controls. Serum levels of adiponectin, visfatin

and especially resitin (positive correlation with Acute Physiology and Chronic Health Evaluation Ⅱ, Ranson and C-reactive protein) are significantly different in mild and severe AP patients, so, they can serve as a markers for the disease severity prediction. Resistin and visfatin can also be used for pancreatic and parapancreatic necrosis prediction, interventions needs and possible, outcome.

Applications

Adipokines could be a useful markers for adipose tissue necrosis. High levels of adipokines could allow for prediction of a severe disease course and outcome even in small pancreatic lesions on computed tomography scans, but further research is needed.

Terminology

AP: Acute inflammatory process of the pancreas with variable involvement of other regional tissues or remote organ systems; Mild AP: Associated with minimal organ dysfunction an uneventful recovery; Severe AP: Associated with organ failure and/or local complications such as necrosis, abscess or pseudocyst; Adipokines: Cytokines secreted by adipose tissue.

Peer review

In the current review, the authors have presented the current knowledge on the role of adipocytokines in predicting severity of AP. The search criteria were scientific and the data has been presented in an easily comprehensible manner.

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S- Editor Gou SX **L- Editor** A **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v18.i45.6628

World J Gastroenterol 2012 December 7; 18(45): 6628-6634
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Trends in the eradication rates of *Helicobacter pylori* infection for eleven years

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Received: June 16, 2012 Revised: October 10, 2012
Accepted: October 22, 2012
Published online: December 7, 2012

Abstract

AIM: To evaluate the trends in the eradication rate of *Helicobacter pylori* (*H. pylori*) over the past 11 years in a single center.

METHODS: This retrospective study covered the period from January 2000 to December 2010. We evaluated 5746 patients diagnosed with gastric ulcers (GU), duodenal ulcers (DU), GU + DU, or nonpeptic ulcers associated with an *H. pylori* infection. We treated them annually with the 2 wk standard first-line triple regimen, proton pump inhibitor (PPI) + amoxicilin + clarithromycin (PAC; PPI, clarithromycin 500 mg, and amoxicillin 1 g, all twice a day). The follow-up test was performed at least 4 wk after the completion of the 2 wk standard *H. pylori* eradication using the PAC regimen. We also assessed the eradication rates of 1 wk second-line therapy with a quadruple standard regimen (PPI *b.i.d.*, tripotassium dicitrate bismuthate 300 mg *q.i.d.*, metronidazole 500 mg *t.i.d.*, and tetracycline 500 mg *q.i.d.*) after the failure of the first-line therapy. Statistical analysis was performed with 95%CI for the differences in the annual eradication rates.

RESULTS: A total of 5746 patients [2333 males (58.8%), 1636 females (41.2%); mean age of males *vs* females 51.31 ± 13.1 years *vs* 52.76 ± 13.6 years, $P < 0.05$, total mean age 51.9 ± 13.3 years (mean ± SD)] were investigated. Among these patients, 1674 patients were excluded: 35 patients refused treatment; 18 patients ceased *H. pylori* eradication due to side effects; 1211 patients had inappropriate indications for *H. pylori* eradication, having undergone stomach cancer operation or chemotherapy; and 410 patients did not undergo the follow-up. We also excluded 103 patients who wanted to stop eradication treatment after only 1 wk due to poor compliance or the side effects mentioned above. Finally, we evaluated the annual eradication success rates in a total of 3969 patients who received 2 wk first-line PAC therapy. The endoscopic and clinical findings in patients who received the 2 wk PAC were as follows: gastric ulcer in 855 (21.5%); duodenal ulcer in 878 (22.1%); gastric and duodenal ulcer in 124 (3.1%), erosive, atrophic gastritis and functional dyspepsia in 2055 (51.8%); and other findings (e.g., MALToma, patients who wanted to receive the therapy even though they had no abnormal endoscopic finding) in 57 (0.5%).

The overall eradication rate of the 2 wk standard first-line triple regimen was 86.5%. The annual eradication rates from 2000 to 2010 were 86.7%, 85.4%, 86.5%, 83.3%, 89.9%, 90.5%, 88.4%, 84.5%, 89.1%, 85.8%, and 88.3%, sequentially (P = 0.06). No definite evidence of a significant change in the eradication rate was seen during the past eleven years. The eradication rates of second-line therapy were 88.9%, 82.4%, 85%, 83.9%, 77.3%, 85.7%, 84.4%, 87.3%, 83.3%, 88.9%, and 84% (P = 0.77). The overall eradication rate of 1 wk quadruple second-line therapy was 84.7%. There was no significant difference in the eradication rate according to the *H. pylori* associated diseases.

CONCLUSION: This study showed that there was no trend change in the *H. pylori* eradication rate over the most recent 11 years in our institution.

Key words: *Helicobacter pylori*; Eradication; Proton pump inhibitor; Therapy; Clarithromycin

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Yoon JH, Baik GH, Sohn KM, Kim DY, Kim YS, Suk KT, Kim JB, Kim DJ, Kim JB, Shin WG, Kim HY, Baik IH, Jang HJ. Trends in the eradication rates of *Helicobacter pylori* infection for eleven years. *World J Gastroenterol* 2012; 18(45): 6628-6634 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i45/6628.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i45.6628

INTRODUCTION

Helicobacter pylori (*H. pylori*) has been one of the most common human infections worldwide and is associated with a number of gastrointestinal diseases, including chronic gastritis, peptic ulcer disease, and gastric malignancy[1]. In multicenter studies, it has been shown that triple therapy with a proton pump inhibitor (PPI), clarithromycin 500 mg and either amoxicillin 1000 mg or metronidazole 500 mg, all taken twice daily, is one of the most effective treatments for *H. pylori* eradication[2]. However, there is no consensus on the length of treatment among the various management guidelines, including the Asia-Pacific consensus guideline[3] and the consensus statements from North America[4] and Europe[5]. European guidelines recommend 1 wk of treatment, whereas in the United States, it is recommended that the triple standard regimen be given for 10-14 d. The second Asia-Pacific consensus guideline recommends a 7-14 d standard regimen; however, it mentions that 14 d triple therapy confers a limited advantage over 7-d triple therapy in *H. pylori* eradication rates. In recent years, a decrease in the eradication success rate of 1 wk of triple therapy has been reported due to antibiotic resistance, especially to clarithromycin[6]. Although clarithromycin resistance is increasing year by year, the current recommended first-line therapy for *H. pylori* infection is PPI, amoxicillin, and clarithromycin for 7-14 d in Korea[7]. The aim of this retrospective observational study was to investigate the trend in the 2 wk PPI-based standard regimen, which included amoxicillin and clarithromycin, under the unfavorable conditions of increasing antibiotic resistance. In addition, we also studied the trend in the eradication success rate of 1 wk second-line therapy that consisted of bismuth-containing quadruple therapy including PPI, metronidazole, and tetracycline.

MATERIALS AND METHODS

Patients

We retrospectively investigated the annual *H. pylori* eradication success rate of patients who visited our hospital from January 2000 to December 2010 and who had been diagnosed as *H. pylori*-infected by at least one positive result from an *H. pylori* culture test, microscopy of a biopsy specimen, or ^{13}C-urea breath test. Patients were excluded due to the following reasons: patient refusal of treatment, abandonment of *H. pylori* eradication treatment, inappropriate indications for *H. pylori* eradication because of a stomach cancer operation or chemotherapy, or follow-up loss after *H. pylori* eradication treatment. We also excluded patients receiving 1 wk PPI + amoxicilin + clarithromycin treatment (PAC; PPI: omeprazole, lansoprazole, pantoprazole, rabeprazole, or esomeprazole, clarithromycin 500 mg, and amoxicillin 1 g, all twice a day) that exhibited poor compliance or adverse effects. We evaluated the success rate of *H. pylori* eradication for all patients who received the 2 wk, first-line standard *H. pylori* eradication PAC regimen. We also evaluated the success rate of eradication of 1 wk bismuth-containing quadruple therapy (PPI *b.i.d*, tripotassium dicitrate bismuthate 300 mg *q.i.d*., metronidazole 500 mg *t.i.d*., and tetracycline 500 mg *q.i.d*.) of patients who failed *H. pylori* eradication treatment by the standard PAC regimen. Among these, patients who did not want treatment, patients who ceased *H. pylori* eradication treatment, and patients lost to follow-up after *H. pylori* eradication treatment were excluded. In the end, we evaluated the success rate of *H. pylori* eradication for a total of 399 patients who received a 1 wk, second-line bismuth-containing quadruple therapy *H. pylori* eradication regimen. This study was approved by the institutional review board of Hallym University Chuncheon Hospital.

Diagnosis of H. pylori infection and assessment of H. pylori eradication

H. pylori infection was defined according to at least one of the following three tests: (1) a positive rapid urease test (CLO test, Delta West, Bentley, Australia) by gastric mucosal biopsy from the lesser curvature of the mid-antrum or

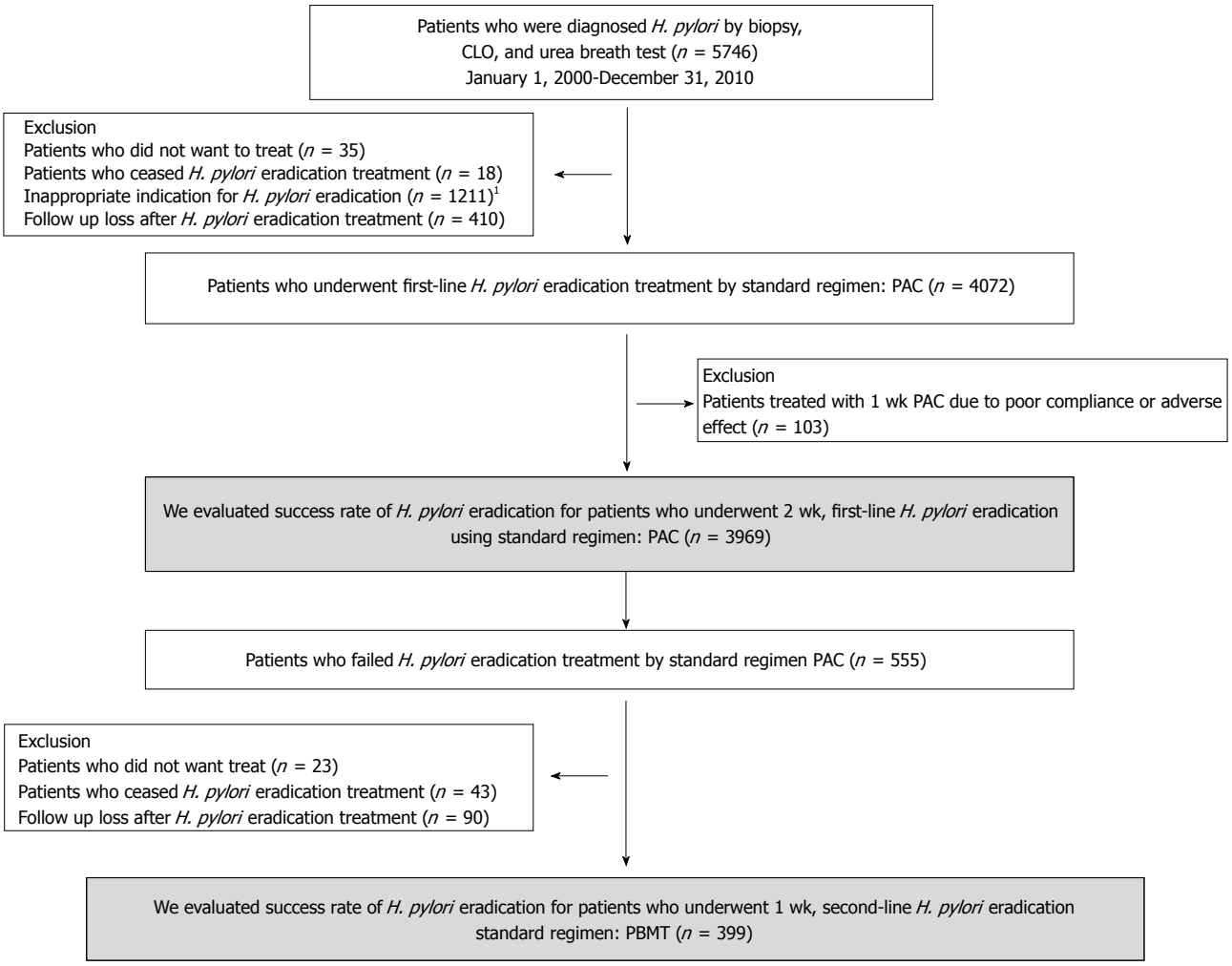

Figure 1 Flow of the study. [1]Inappropriate indications for *Helicobacter pylori* (*H. pylori*) eradication: Stomach cancer operation or chemotherapy. PAC: Proton pump inhibitor *b.i.d.* + amoxicillin 1 g *b.i.d.* + clarithromycin 500 mg; PBMT: Proton pump inhibitor *b.i.d* + tripotassium dicitrate bismuthate 300 mg *q.i.d.* + metronidazole 500 mg *t.i.d.* + tetracycline 500 mg *q.i.d.*

mid-body; (2) histological evidence of *H. pylori* by modified Giemsa staining in the lesser and greater curvature of the mid-antrum or mid-body, respectively; or (3) a positive C-urea breath test. *H. pylori* eradication was defined as a negative ^{13}C-urea breath test or a combination of the rapid urease test, Giemsa staining, and culture when follow-up endoscopy was necessary. The follow-up test was performed at least 4 wk after the completion of the 2 wk standard *H. pylori* eradication using the PAC regimen.

Statistical analysis

Statistical analysis was performed with 95%CI for the differences in the annual eradication rates from January 2000 to December 2010. Continuous variables were analyzed by Student's *t* test, and categorical variables, by the χ^2 test or Fisher's exact test. A P value of < 0.05 was considered to be statistically significant.

RESULTS

A total of 5746 patients [2333 males (58.8%), 1636 females (41.2%); mean age of males *vs* females 51.31 ± 13.1 years *vs* 52.76 ± 13.6 years, $P < 0.05$, total mean age 51.9 ± 13.3 years (mean ± SD)] were retrospectively investigated for the period of January 2000 to December 2010 in this study. The retrospective assessment flow is summarized in Figure 1. Among these patients, 1674 patients were excluded: 35 patients refused treatment; 18 patients ceased *H. pylori* eradication treatment due to side effects, such as abdominal discomfort, diarrhea, taste disturbance, or nausea; 1211 patients had inappropriate indications for *H. pylori* eradication therapy, having undergone a stomach cancer operation or chemotherapy; and 410 patients did not undergo the follow-up assessment for *H. pylori* eradication after the 2 wk PAC treatment. We also excluded 103 patients who wanted to stop the eradication treatment after only 1 wk due to poor compliance or the side effects mentioned above. Finally, we evaluated the annual eradication success rates in a total of 3969 patients who received 2 wk PAC therapy. Demographic characteristics are summarized in Table 1. Of the patients included in the study, 735 (18.5%) were current smokers, and 28.7%

Table 1 Baseline characteristics of the *Helicobacter pylori* eradication population

Baseline characteristics	Data *n* (%)
Sex	
Male	2333 (58.8)
Female	1636 (41.2)
Age (yr, mean ± SD)	51.9 ± 13.3
Current smoker	735 (18.5)
Alcohol intake	1140 (28.7)
Endoscopic and clinical diagnosis	
GU	855 (21.5)
DU	878 (22.1)
GU + DU	124 (3.1)
Non-ulcer dyspepsia[1]	2055 (51.8)
Other[2]	57 (1.4)

[1]Functional dyspepsia, erosive gastritis, atrophic gastritis; [2]Gastric cancer, MALToma. GU: Gastric ulcers; DU: Duodenal ulcers.

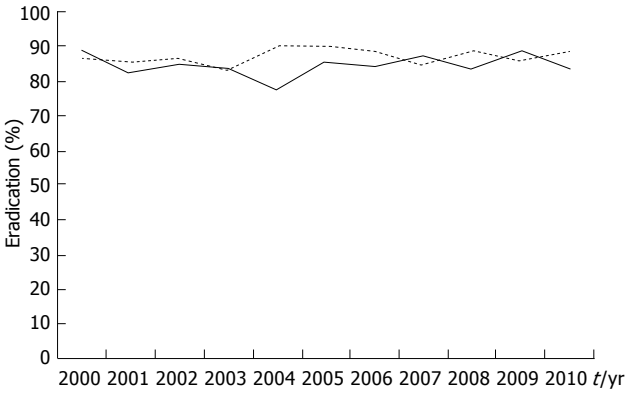

Figure 2 Efficacy of 2 wk first-line (a dotted line) proton pump inhibitor *b.i.d.* + amoxicillin 1 g *b.i.d.* + clarithromycin 500 mg and 1 wk second-line (a solid line) bismuth-containing quadruple therapy for the eradication of *Helicobacter pylori* by year.

were alcohol drinkers. Endoscopic and clinical findings in patients who received the 2 wk PAC were as follows: gastric ulcer in 855 (21.5%); duodenal ulcer in 878 (22.1%); gastric and duodenal ulcer in 124 (3.1%), erosive, atrophic gastritis and functional dyspepsia in 2055 (51.8%), and other findings (e.g., MALToma, patients who wanted to receive the therapy even though they had no abnormal endoscopic finding) in 57 (1.4%). When endoscopy is not indicated, C^{13} urea breath tests (3030, 76.3%), biopsies (22, 0.6%), and CLO test (503, 12.7%) are accepted to determine the outcome of *H. pylori* eradication therapy. In some cases, biopsy + CLO test (329, 8.2%) or CLO test + C^{13} urea breath test was used for the diagnosis of *H. pylori* eradication therapy. Successful eradication rates for each year were follows: 2000, 86.7%; 2001, 85.4%; 2002, 86.5%; 2003, 83.3%; 2004, 89.9%; 2005, 90.5%; 2006, 88.4%; 2007, 84.5%; 2008, 89.1%; 2009, 85.8%; and 2010, 88.3% (P = 0.06). Figure 2 summarizes the annual eradication rates year by year from 2000 to 2010. The overall eradication rate was 86.5%, that is, 3435 of 3969 patients who received the 2 wk PAC (95%CI: 85.4% to 87.6%). The P value was 0.09. The annual eradication rates of the 2 wk PAC regimen revealed a relatively constant rate over the years. According to endoscopic and clinical findings, the eradication rates were not significantly different by year. We also investigated the eradication rates of 1 wk bismuth-containing quadruple therapy for 555 patients who failed the *H. pylori* eradication treatment using the 2 wk PAC therapy. Among the 555 patients, 156 patients were excluded for the following reasons: 23 patients declined treatment, 43 patients ceased *H .pylori* eradication treatment due to poor compliance or side effects such as diarrhea, nausea, vomiting, or stool color change, and 90 patients were lost to follow-up after *H. pylori* eradication treatment. Finally, we found the rates of successful eradication in 399 patients who received 1 wk bismuth-containing quadruple second-line therapy for each year to be as follows: 2000, 88.9%; 2001, 82.4%; 2002, 85.0%; 2003, 83.9%; 2004, 77.3%; 2005, 85.7%; 2006, 84.4%; 2007, 87.3%; 2008, 83.3%; 2009, 88.9%; and 2010, 84.0% (P = 0.77). Figure 2 summarizes the annual eradication rates year by year from 2000 to 2010. The overall eradication rate was 84.7%, and the rate among those who received 1 wk second-line therapy was 338 of 399 patients (95%CI: 80.9% to 87.9%). The P value was 0.07. The annual eradication rates of the 1 wk second-line therapy revealed a relatively constant rate over the years.

DISCUSSION

The PAC regimen is one of the most widely used therapies for the first-line treatment of *H. pylori* infection. A recent study reported the eradication rate of *H. pylori* with one wk standard triple therapy to be 75% in Korea[8]. There is still no general consensus regarding the optimal duration of triple therapy for *H. pylori* eradication, as mentioned above. A recent large, multicenter, double-blind, randomized study concluded that the 1 wk and 2 wk standard triple regimens for *H. pylori* eradication are similar in terms of efficacy, safety, and patient compliance[9]. Two meta-analyses have reported that 2 wk triple therapy achieves considerably better results than 1 wk therapy[10,11]. Recently, many studies have reported that the efficacy of the standard triple regimen has decreased[12-14] because of the increased antibiotic resistance rate; that is, the standard triple regimen of PPI, amoxicillin, and clarithromycin administered to patients infected with the clarithromycin-resistant strain was not successful[15]. Although the general consensus is that a 1 wk PAC is preferable, we have treated patients infected by *H. pylori* with the 2 wk PAC to overcome increasing antibiotic resistance. Our data on the 2 wk PAC regimen shows a relatively constant rate over the year from 2000 to 2010. A recent chronological analysis of the results of meta-analyses performed between 1998 and 2010 showed that first-line standard triple regimens achieved eradication rates of around only 80% (intention-to-treat)[16]. Given that the overall eradication rate in this study was 86.5% (3435 of 3969 patients who received the 2 wk PAC), we concluded that the 2 wk PAC regimen had an accept-

able efficacy for *H. pylori* eradication. Many investigators have commented that the decreasing eradication rate using standard triple therapy is due to increasing antibiotic resistance. However, a number of factors, including resistance, which varies widely, influence the success of antibiotic regimens. The resistance rates of antibiotics that are widely used in eradication therapy, including amoxicillin and tetracyclines, are relatively lower than clarithromycin and metronidazol[15]. Therefore, the true challenge for clinical practice lies mainly in resistance to metronidazole or clarithromycin. In Korea, the rates of resistance to clarithromycin and metronidazole were reported from 1.6% up to 29.7% and from 35.7% up to 49.6 % from 1996 to 2006, respectively[17]. This is a major factor in the reduced effectiveness of triple regimens containing clarithromycin[18]. Clarithromycin must bind to ribosomes to kill *H pylori*. Resistance is associated with failure to bind to ribosomes, such that resistance cannot be overcome by increasing the dose or duration[15]. However, patients do not always have perfect compliance. Because *H. pylori* treatment failures may also occur independently of resistance, that is, treatment may fail but the organism remains susceptible to the antibiotic[19], we concluded that the 2 wk standard PAC regimen may be fit for use in eradicating *H. pylori*. Bacteria oscillate between a phenotypically resistant and a phenotypically susceptible state, during which they can be eradicated. To extend the duration of treatment such that the antibiotic will be present during at least one period of susceptibility may be an alternative option to overcome antibiotic resistance[20]. Consistent with the results from Korea in this study, several authors have reported an increasing trend in clarithromycin resistance rates in other areas, such as the United States[21], Turkey[22], *etc*. Nevertheless, exceptional cases where clarithromycin resistance rates have remained stable have also been reported[23]. These results strongly suggest that there is an institutional and geographical difference in the antibiotic resistance of *H. pylori*. This fact and our results in this study suggest that the 2 wk PAC regimen may be effective in eradicating *H. pylori* in some countries or limited geographical areas. In a recent review, the eradication rates of quadruple therapy were 75%-95%[24]. In Korea, where the resistance to metronidazole is high, the eradication rates of second line quadruple therapy have been reported to be 54.5%-76.7% and 70.4%-83.9% in intention to treat and per protocol analysis[25,26], respectively, based on studies in which the therapeutic durations varied from 7 to 14 d. Metronidazole is a prodrug that is activated by *H. pylori* enzymes to become active within the cell. There are a number of different enzyme pathways that can accomplish this task, and clinically, by increasing the dose and duration, it is possible to overcome, at least partially, metronidazole resistance[2,27]. Thus, there is the possibility that quadruple therapy might be more effective with after a treatment duration of longer than 1 wk. However, some studies have shown the benefit of eradication with prolonged treatment durations[28], while others have not[29,30]. We previously reported that 1 wk bismuth-containing quadruple therapy can be as an effective as 2 wk therapy after the failure of the first-line eradication therapy. In this study, the eradication rates of 1 wk bismuth-containing quadruple therapy have no significant differences from the consecutive, annual eradication rates, in spite of increasing metronidazole resistance in Korea. Therefore, although 1 wk bismuth-containing quadruple therapy is not effective up to more than 90% yet, we concluded that 1 wk bismuth-containing quadruple therapy can be used to treat patients who failed the first *H. pylori* eradication. To raise the eradication rate of *H. pylori*, when the clarithromycin resistance rate is higher than 20%, it is recommended that drug sensitivity tests be carried out prior to eradication[5]. However, there are several limitations to performing a culture before the first-line treatment for *H. pylori* infection. Cultures are expensive, owing to the costs of the endoscopic procedures, and they are time-consuming. Therefore, cultures are not always available on a routine basis. Until now, cultures for *H. pylori* have mainly been used to perform epidemiological and pharmacologic research. Because extending the duration of the first line PAC regimen from 1 wk to 2 wk may improve the efficacy of the *H. pylori* eradication rate, the 2 wk PAC regimen is preferable for treating *H. pylori*. However, this study does have limitations. One is that it is a retrospective, observational study. Therefore, there was some bias, such as an uneven diagnostic method for the determination of the outcome of *H. pylori* eradication therapy in each year. The other endoscopic findings were not even taken in each year. Additionally, we could not obtain the data on antibiotic resistance including amoxicillin, clarithromycin, metronitazole, and tetracyclin in accordance with the eradication rate.

In conclusion, we show the efficacy of a 2 wk PAC regimen and 1 wk bismuth-containing quadruple therapy has not changed across the 2000 to 2010 period in South Korea. The efficacy of 2 wk PAC and 1 wk quadruple second line therapy is by no means acceptable and satisfactory.

COMMENTS

Background

In recent years, a decrease in the eradication success rate of 1 wk of triple therapy has been reported due to antibiotic resistance, especially to clarithromycin. Although clarithromycin resistance is increasing year by year, the currently recommended first-line therapy for *Helicobacter pylori* (*H. pylori*) infection is proton pump inhibitor (PPI), amoxicillin, and clarithromycin for 7-14 d in South Korea. This retrospective, observational study intended to investigate the trend in the 2 wk PPI-based standard regimen including amoxicillin and clarithromycin under the unfavorable conditions of increasing antibiotic resistance.

Research frontiers

The overall eradication rate of the 2 wk standard first-line triple regimen from 2000 to 2010 was 86.5%. No definite evidence of a significant change in the eradication rate was seen during the past eleven years. The overall eradication rate of 1 wk bismuth-containing quadruple, second-line therapy from 2000 to 2010 was 84.7%. The annual eradication rates of the 1 wk second-line therapy revealed a relatively constant rate over the year.

Innovations and breakthroughs

Authors show that the efficacy of 2 wk PPI + amoxicilin + clarithromycin (PAC)

regimen and 1 wk bismuth-containing quadruple therapy did not change over the period from 2000 to 2010 in South Korea. The efficacy of the 2 wk PAC and 1 wk quadruple second line therapy is by no means acceptable and satisfactory.

Applications

Authors conclude that the 2 wk standard PAC regimen may be fit for use in eradicating *H. pylori*. Bacteria oscillate between a phenotypically resistant and a phenotypically susceptible state, during which they can be eradicated. To extend the duration of treatment such that the antibiotic will be present during at least one period of susceptibility may be an alternative option to overcome antibiotic resistance.

Peer review

In this study, the authors retrospectively evaluated the eradication rate of *H. pylori* in a single center in the Republic of Korea for the period of January 2000 to December 2010. In 3969 patients who received a two weeks standard first-line triple therapy, an overall eradication rate of 86.5% has been found, with no significant ($P = 0.09$) difference in the annual eradication rate during the eleven years. Furthermore, in the 399 patients who failed *H. pylori* eradication and received a 1-wk second-line therapy, an overall eradication rate of 84.7% has been found, with a relatively constant rate over the years. The authors conclude that there was no trend of change in the *H. pylori* eradication rate over the last 11 years in their institution and that a 2-wk first-line and a 1-wk second-line therapy can still be used in Korea.

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S- Editor Gou SX **L- Editor** A **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v18.i45.6635

World J Gastroenterol 2012 December 7; 18(45): 6635-6644
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

UGT1A1 predicts outcome in colorectal cancer treated with irinotecan and fluorouracil

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Supported by National Natural Science Foundation Project, No. 30971579; the Capital Medical Development Foundation, No. 2007-2029
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Received: May 16, 2012 Revised: September 13, 2012
Accepted: October 22, 2012
Published online: December 7, 2012

Abstract

AIM: To evaluate effects of UDP-glucuronosyltransferase1A1 (*UGT1A1*) and thymidylate synthetase (*TS*) gene polymorphisms on irinotecan in metastatic colorectal cancer (mCRC).

METHODS: Two irinotecan- and fluorouracil-based regimens, FOLFIRI and IFL, were selected as second-line therapy for 138 Chinese mCRC patients. Genomic DNA was extracted from peripheral blood samples before treatment. *UGT1A1* and *TS* gene polymorphisms were determined by direct sequencing and restriction fragment length polymorphism, respectively. Gene polymorphisms of UGT1A1*28, UGT1A1*6 and promoter enhancer region of TS were analyzed. The relationship between genetic polymorphisms and clinical outcome, that is, response, toxicity and survival were assessed. Pharmacokinetic analyses were performed in a subgroup patients based on different *UGT1A1* genotypes. Plasma concentration of irinotecan and its active metabolite SN-38 and inactive metabolite SN-38G were determined by high performance liquid chromatography. Differences in irinotecan and its metabolites between *UGT1A1* gene variants were compared.

RESULTS: One hundred and eight patients received the FOLFIRI regimen, 29 the IFL regimen, and one irinotecan monotherapy. One hundred and thirty patients were eligible for toxicity and 111 for efficacy evaluation. One hundred and thirty-six patients were tested for UGT1A1*28 and *6 genotypes and 125 for promoter enhancer region of TS. Patients showed a higher frequency of wild-type UGT1A1*28 (TA6/6) compared with a Caucasian population (69.9% *vs* 45.2%). No significant difference was found between response rates and UGT1A1 genotype, although wild-type showed lower response rates compared with other variants (17.9% *vs* 24.2% for UGT1A1*28, 15.7% *vs* 26.8% for UGT1A1*6). When TS was considered, the subgroup with homozygous UGT1A1*28 (TA7/7) and non-3RG genotypes showed the highest response rate (33.3%), while wild-type UGT1A1*28 (TA6/6) with non-3RG only had a 13.6% response rate, but no significant difference was found. Logistic regression showed treatment duration was closely linked to clinical response. In toxicity comparison, UGT1A1*28 TA6/6 was associated with lower incidence of grade 2-4 diarrhea (27.8% *vs* 100%), and significantly reduced the risk of grade 4 neutropenia compared with TA7/7 (7.8% *vs* 37.5%).

Wild-type UGT1A1*6 (G/G) tended to have a lower incidence of grade 3/4 diarrhea *vs* homozygous mutant (A/A) genotype (13.0% *vs* 40.0%). Taking *UGT1A1* and *TS* genotypes together, lower incidence of grade 2-4 diarrhea was found in patients with non-3RG *TS* genotypes, when TA6/6 was compared with TA7/7 (35.3% *vs* 100.0%). No significant association with time to progression (TTP) and overall survival (OS) was observed with either *UGT1A1* or *TS* gene polymorphisms, although slightly longer TTP and OS were found with UGT1A1*28 (TA6/6). Irinotecan PK was investigated in 34 patients, which showed high area under concentration curve (AUC) of irinotecan and SN-38, but low AUC ratio (SN-38G / SN-38) in those patients with UGT1A1*28 TA7/7.

CONCLUSION: A distinct distribution pattern of *UGT1A1* genotypes in Chinese patients might contribute to relatively low toxicity associated with irinotecan and 5-fluorouracil in mCRC patients.

Key words: Irinotecan; Fluorouracil; UDP-glucuronosyltransferase1A1; Thymidylate synthetase; Polymorphisms; Pharmacokinetics; Treatment outcome; Toxicity; Metastatic colorectal cancer

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Wang Y, Shen L, Xu N, Wang JW, Jiao SC, Liu ZY, Xu JM. *UGT1A1* predicts outcome in colorectal cancer treated with irinotecan and fluorouracil. *World J Gastroenterol* 2012; 18(45): 6635-6644 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i45/6635.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i45.6635

INTRODUCTION

The current management of metastatic colorectal cancer (mCRC) uses fluorouracil-based regimens in combination with either oxaliplatin or irinotecan. These regimens mainly differ in their toxicity profiles with neutropenia and late diarrhea being associated with irinotecan-based therapy versus neurotoxicity with oxaliplatin-based treatment.

In our previous studies on the relationship of UDP-glucuronosyltransferase1A1 (*UGT1A1*) polymorphisms and irinotecan-related diarrhea, we found that UGT1A1*28 genotype was significantly associated with the occurrence of diarrhea, while the polymorphisms of UGT1A7 and UGT1A9 variants were found to be unrelated. Mutated *UGT1A1*28* genotype was seen infrequently in the Chinese population, therefore, this might explain the lower incidence of late diarrhea in our patients treated with irinotecan compared to that in the Caucasian population[1,2]. However, the role of UGT1A in clinical response to irinotecan- and 5-fluorouracil (5-FU)-based treatment, non-diarrhea toxicities and prognosis remains unclear. In addition, thymidylate synthetase (TS), the enzyme targeted by 5-FU, deserves more attention because most patients that receive irinotecan are treated with irinotecan and 5-FU in combination.

UGT1A polymorphisms have become the focus of irinotecan pharmacokinetics and toxicity research because they are involved in metabolism of cytotoxic SN-38 (an active metabolite of irinotecan) to inactive SN-38 glucuronide (SN-38G) (Figure 1). Di Paolo *et al*[3] have demonstrated that UGT1A1 polymorphisms are closely correlated with glucuronidation rates, and patients with higher concentration of SN-38G are less susceptible to irinotecan-induced toxicity. Although, several clinical trials have confirmed that patients carrying different genotypes of UGT1A1 had varied degrees of tolerance to irinotecan, it is still unclear whether UGT1A1 has any influence on treatment efficacy. Three studies tested if UGT1A1 isoforms had any impact on treatment outcome, however, their conclusions were inconsistent[4-6].

Irinotecan is often combined with 5-FU in mCRC treatment, therefore, 5-FU should be taken into consideration for response and toxicity evaluation as well. *In vivo*, the active metabolite of 5-FU inhibits TS activity by forming complexes with TS and 5, 10-methylene-tetrahydrofolate (Figure 1)[7]. Evidence supports the determinant role of TS promoter region polymorphism in 5-FU treatment efficacy and tolerance[8,9]. Given higher accuracy and consistency in testing polymorphisms, tremendous efforts have been put into using TS polymorphism as a genetic marker for predicting clinical response, toxicity and prognosis in mCRC patients treated with 5-FU[10-13].

This study aimed to evaluate the effects of UGT1A1/TS polymorphisms in Chinese mCRC patients treated with irinotecan and fluorouracil including toxicity and clinical outcome.

MATERIALS AND METHODS

Drug administration

Two regimens were selected for this study: (1) FOLFIRI: irinotecan (Camptosar; Pfizer, United States) 180 mg/m^2 90-min *i.v.* infusion on day 1; leucovorin 200 mg/m^2 *i.v.* infusion on days 1 and 2; followed on days 1 and 2 by 5-FU 400 mg/m^2 *i.v.* bolus, then 600 mg/m^2 *i.v.* over 22 h continuous infusion; repeated every 2 wk; (2) IFL: irinotecan 125 mg/m^2 as a 90-min *i.v.* infusion on days 1, 8, 15 and 22; leucovorin 20 mg/m^2 *i.v.* infusion on days 1, 8, 15 and 22; 5-FU 500 mg/m^2 *i.v.* bolus on days 1, 8, 15 and 22; every 6 wk.

Patient eligibility

The criteria for inclusion were: at least 18 years old; histologically confirmed mCRC; failed or intolerant to oxalipl-

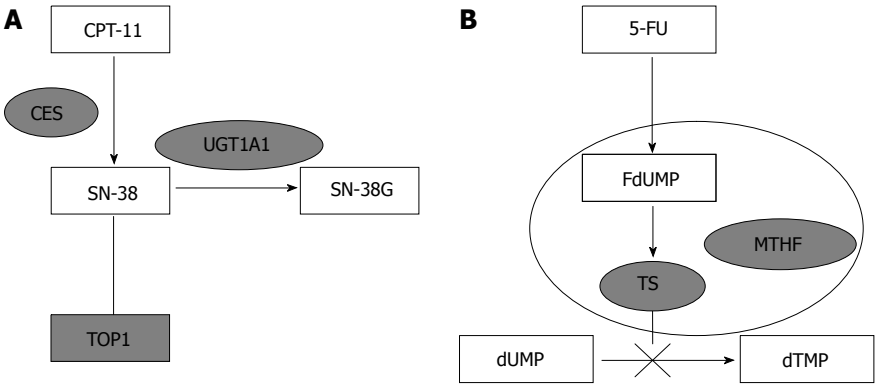

Figure 1 Schematic diagram of UDP-glucuronosyltransferase1A1 and thymidylate synthetase. A: UDP-glucuronosyltransferase1A1 (UGT1A1) is the main enzyme involved in the glucuronidation of SN-38 (SN-38G). Single-nucleotide polymorphisms (SNPs) of UGT1A1 are the key factor in irinotecan metabolism; B: Thymidylate synthetase (TS) is the main target of 5-fluorouracil (5-FU). The ternary complex of TS, active metabolite of 5-FU (FdUMP) and methyl-tetra-hydrofolic acid (MTHF) inhibits DNA synthesis. SNP of TS affects the expression of enzyme and 5-FU efficacy. CES: Carboxylesterases; TOP1: Topoisomerase-1.

atin-based regimens; the Eastern Cooperative Oncology Group (ECOG) performance status 0-2; no chemotherapy at least 4 wk before study enrollment; life expectancy > 3 mo; neutrophil count $\geqslant 1.5 \times 10^9$/L, platelet count $\geqslant 8 \times 10^{10}$/L, serum creatinine $\leqslant$ 1.25 upper limit normal (ULN), total bilirubin $\leqslant$ 1.25 ULN, alanine aminotransferase and aspartate aminotransferase $\leqslant$ 2.5 ULN ($\leqslant$ 5 ULN with liver metastasis); normal electrocardiogram. Written informed consent was required and the study was performed in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. All patients' information and samples were collected from trial participating centers.

Evaluation of response and toxicity

Tumor response was assessed by RECIST 1.0[14] based on the results of computed tomography. Time to progression (TTP) was defined as the time from the start of treatment to the date of progression. Overall survival (OS) was defined as the time from the start of the treatment to the date of death. Toxicity was assessed according to the NCI-CTC 3.0[15].

Genomic DNA extraction, polymerase chain reaction and genotyping assay

Two-milliliter peripheral blood samples were collected before starting treatment and frozen at -20 ℃. Genomic DNA was extracted from these samples using the Peripheral Blood Extraction Kit (Tiangen, China). The fragments of UGT1A1*28 and UGT1A1*6 were amplified by polymerase chain reaction (PCR). The Primer 5.0 software was used to design the sense primer (5'-GCCAGTTCAACTGTTGTTGC-3') and antisense primer (5'-GTCCGTCAGCATGACATCAA-3'). Each 25-μL PCR reaction mixture included 5 ng DNA template, 1 mmol/L dNTPs, 0.4 mmol/L $MgSO_4$, 0.25 μmol/L primers, 0.5 U KOD-plus polymerase (TOYOBO, Japan) and 10 × KOD plus buffer. The PCR profile included 2.5 min denaturation at 94 ℃, 35 cycles of 30 s at 94 ℃, 60 s at 57 ℃, 60 s at 68 ℃, with a final 7-min extension at 68 ℃. The PCR products were sequenced by ABI-3730 DNA analyzer and all single-nucleotide polymorphisms (SNPs) were analyzed by Polyphred 5.04 with additional manual proofreading.

Restriction fragment length polymorphism testing for promoter enhancer region of TS

Promoter enhancer region of 2R or 3R and G>C SNP in 3R were selected for testing. PCR-based restriction fragment length polymorphism was applied to detect these variants. The PCR conditions were same as described above with sense primer of 5'-GTGGCTCCTGCGTTTCCCCC-3' and antisense primer of 5'-GCTCCGAGCCGGCCACAGGCATGGCGCGG-3'. The PCR profile included initial 15 cycles with annealing temperature at 63 ℃, and another 30 cycles with annealing temperature at 62 ℃. The PCR products were electrophoresized in 3% agarose gel to differentiate 215-bp 2R genotype and 243-bp 3R genotype. 3R gene polymorphism was detected *via* electrophoresis after enzyme digestion. After *Hae*Ⅲ treatment, 3RG genotype was separated to fragments of 66, 47, 45, 44, 28 and 13 bp, whereas 3RC genotype was separated to fragments of 94, 47, 45, 44 and 13 bp. The 20-μL reaction system contained 1 μL *Hae* Ⅲ Takara), 2 μL 10 × M buffer, 17 μL purified PCR products for 37 ℃ overnight incubation.

Pharmacokinetics study

Five-milliliter heparinized blood samples were collected before administration of irinotecan, 1 and 1.5 h during infusion, and 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 36, 48, 60 and 72 h after termination of infusion. Plasma samples were obtained by 3000 r/min centrifugation for 10 min and kept at -40 ℃. The concentrations of the drug and its metabolites were determined by high performance liquid chromatography with postcolumn fluorescence derivatization (HPLC-FLD). Two hundred microliters plasma was added to 50 μL 1 ng/mL camptothecin, 150 μL methanol, and 200 μL acetonitrile, and vortexed for 2 min. The mixture was then centrifuged at 15 000 r/min for 10 min. Supernatant (200 μL) was placed with 100 μL 1 mol/L HCl before HPLC analysis. Agilent 1100 chromatographic system consisted of quaternary pumps, an automatic sample injection system, fluorescence detector, and Znertsil ODS-C18 Column. The mobile phases were eluted through the column and contained acetonitrile-sterile water (75:75 by volume) and phosphate buffer at pH 4. Irinotecan was kindly donated by Pfizer and camptothecin was provided by National Institute for the Control of Pharmaceutical and Biological Products. SN-38 was synthesized by Shanghai Zhangjiang Biochemical

Table 1 Association of UDP-glucuronosyltransferase1A1 and thymidylate synthetase genotypes with tumor response

Genotypes	Tumor response	
	n (%)	*P* value[1]
UGT1A1*28		
TA 6/6	14 (17.9)	0.446
TA 6/7, TA 7/7	8 (24.2)	
UGT1A1*6		
G/G	11 (15.7)	0.217
G/A, A/A	11 (26.8)	
TS promotor		
3RG/3RG	3 (17.6)	0.880
3RG/3RC, 3RG/2RG	5 (23.8)	
3RC/3RC, 3RC/2RG, 2RG/2RG	12 (19.4)	

[1]Fisher's exact test for all genotypes. Tumor response including complete and partial response, 111 patients were assessable for tumor response. UGT1A1: UDP-glucuronosyltransferase1A1; TS: Thymidylate synthetase.

Company. Blank human plasma was obtained from Blood bank of 307 Hospital. The lowest limit of quantification of irinotecan, SN-38 and SN-38G were 50 ng/mL, 1.25 ng/mL and 5 ng/mL, respectively, with within-day and between-day imprecision < 15%. C_{max} and T_{max} were observed values. $AUC_{0\rightarrow t}$ was acquired by linear trapezoidal approximation. $AUC_{0\rightarrow\infty} = AUC_{0\rightarrow t} + Ct/ke$.

Statistical analysis

The χ^2 test was used to determine if the allele frequencies were matched to Hardy-Weinberg equilibrium. Associations between genotypes, clinical response and toxicity in Chinese and Caucasian populations were assessed by using Fisher's exact test and Cochran-Armitage trend test. Kaplan-Meier estimates and the log-rank test were used in TTP and OS analysis. Cox regression and logistic regression models were examined in multivariate analysis with and without time variables, respectively. The linkage between genotypes and pharmacokinetic parameters was analyzed by Mann-Whitney *U* and Kruskal-Wallis tests. Two-sided tests were used to determine statistically significant *P* values ($P < 0.05$) using SPSS 13.0 software.

RESULTS

Between September 2005 and April 2009, 138 mCRC patients were enrolled. Of these, 130 patients were eligible for toxicity evaluation and 111 qualified for treatment efficacy evaluation. Of 138 patients, 108 received the FOLFIRI regimen, 29 the IFL regimen, and one irinotecan monotherapy. The median age of patients was 52 years (range: 26-81 years) with 63% being male. One hundred and eleven patients were followed until cancer progression or death. The overall response rate (complete + partial response) was 19.5%. The median TTP was 5.6 mo (95%CI: 3.9-7.3) and the median OS was 17 mo (95%CI: 11.9-22.1). The incidence of grade 3/4 neutropenia was 29.5%, and 17.4% participants developed grade 3/4 late diarrhea. However, the incidences in the FOLFIRI regimen were decreased, with 21.8% grade 3/4 neutropenia (95%CI: 13.7%-29.9%) and 10.9% grade 3/4 late diarrhea (95%CI: 4.8%-16.9%).

Table 2 Association of UDP-glucuronosyltransferase1A1 in combination with thymidylate synthetase genotypes with tumor response

UGT1A1*28	TS promoter		
	3RG/3RG	3RG/3RC, 3RG/2RG	3RC/3RC, 3RC/2RG, 2RG/2RG
TA6/6	2/11 (18.1)	4/13 (30.8)	6/44 (13.6)
TA6/7	1/4 (25)	1/6 (16.7)	5/15 (33.3)
TA7/7	0/2 (0)	0/2 (0)	1/3 (33.3)

Tumor response including complete and partial response, 111 patients were assessable for tumor response, no significant difference was found (Fisher's exact test). UGT1A1: UDP-glucuronosyltransferase1A1; TS: Thymidylate synthetase.

UGT1A1 and TS polymorphisms correlation with clinical response

We tested UGT1A1 genotypes in 136 patients and TS promoter enhancer region in 125 (Figure 2). We observed that the response rates of UGT1A1*28 alleles TA6/6 and TA6/7 + TA7/7 were 17.9% and 24.2%, respectively, and those of UGT1A1*6 alleles G/G and G/A + A/A were 15.7% and 26.8%, respectively, without statistical significance observed.

Based on mRNA transcriptional activity, TS was categorized into 3RG/3RG homozygous group with highest activity, 3RG heterozygous group (3RG/3RC and 3RG/2RG) with median activity, and non-3RG group (3RC/3RC, 3RC/2RG and 2RG/2RG) with lowest activity. Although not statistically significant, 3RG/3RG homozygous group was found with lowest response rate (17.6%) (Table 1). Taking *UGT1A1* and *TS* genotypes together, the subgroup with TA7/7 and non-3RG genotypes showed the highest response rate of 33.3%. Furthermore, under the same non-3RG background, no significant difference in response rate was found between TA6/6 and TA7/7 (13.6% *vs* 33.3%, $P = 0.154$) (Table 2). A multivariate logistic regression model was fitted to our data. The covariates included: chemotherapy regimen, treatment duration, ECOG performance status, sex, age, *UGT1A1* genotypes, and TS promoter enhancer region, but only treatment duration (odds ratio 1.268, 95%CI: 1.027-1.565, $P = 0.027$) was closely linked to clinical response.

UGT1A1 polymorphisms correlation with toxicity

The enzyme activities of UGT1A1*28 alleles from highest to lowest were TA6/6, TA6/7 and TA7/7. TA7/7 showed the highest incidence of grade 2-4 late diarrhea ($P < 0.0005$). Compared with TA6/6, the risk ratio of TA6/7 and TA7/7 was 2.6 (95%CI: 1.20-5.63). Although not statistically significant, a trend of higher incidence of grade 3/4 diarrhea was observed in patients with lower activity genotypes. A similar finding was obtained in the UGT1A1*6 study. G/G with the highest activity was associated with lowest incidence of grade 3/4 diarrhea (trend

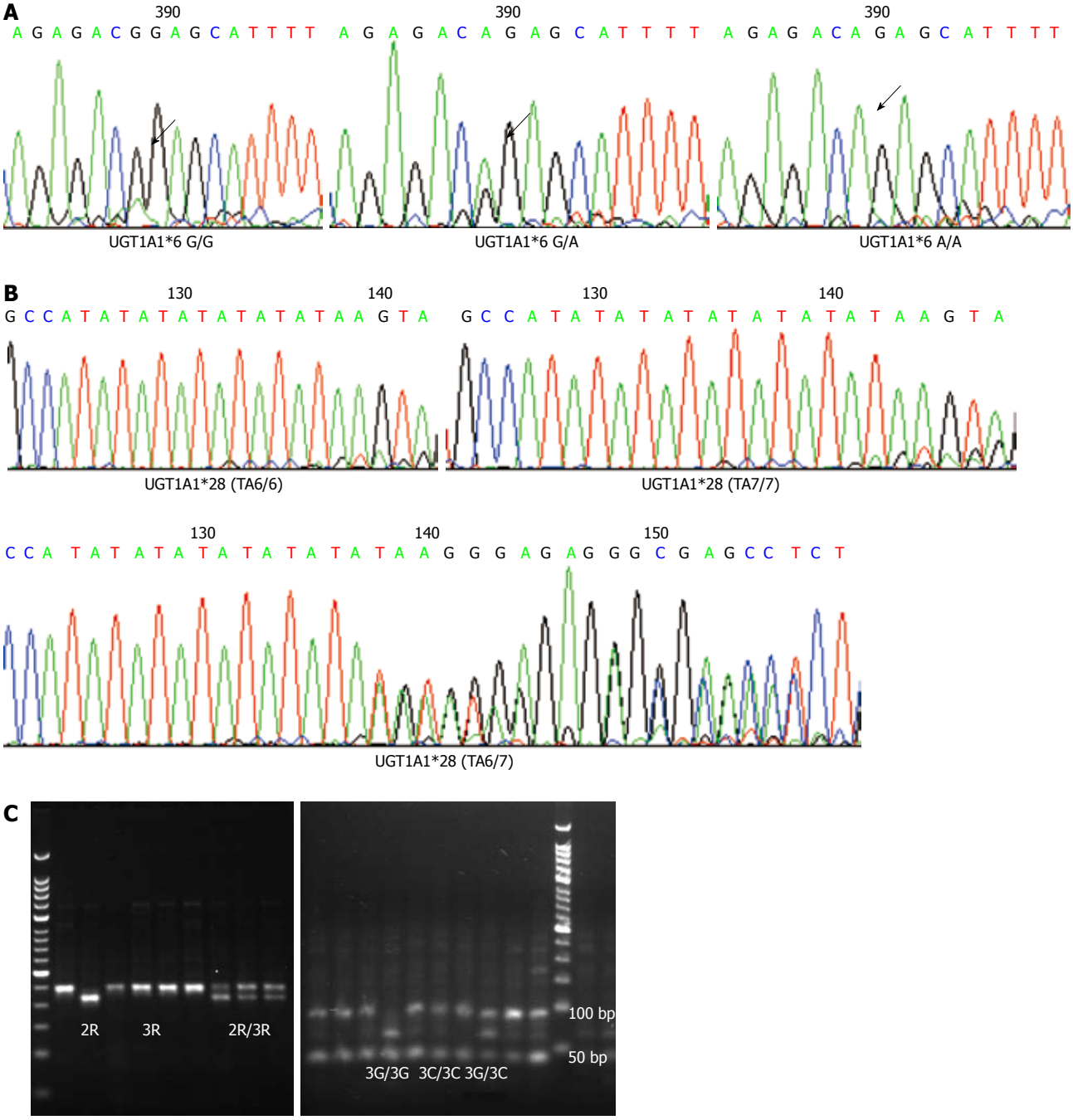

Figure 2 Analysis of UDP-glucuronosyltransferase1A1*28 and *6 by DNA sequencing and thymidylate synthetase by restriction fragment length polymorphism. A: 211G>A of UDP-glucuronosyltransferase1A1 (UGT1A1)*6; B: (TA) nTAA variant of UGT1A1*28; C: Polymerase chain reaction-based restriction fragment length polymorphism for 2R or 3R and G>C single-nucleotide polymorphism in 3R located in the 5'-UTR region of the thymidylate synthetase gene.

test $P = 0.057$). Compared with G/G, the risk ratio of grade 3/4 diarrhea in patients with G/A and A/A was 2.18 (95%CI: 0.87-5.42) (Table 3). In addition, UGT1A1*28 showed a similar correlation pattern with neutropenia. Grade 4 neutropenia was dominantly observed in TA7/7 patients ($P = 0.043$, trend test, $P = 0.017$). Compared with TA6/6, the risk ratio of grade 3/4 neutropenia in patients carrying TA6/7 and TA7/7 was 1.75 (95%CI: 0.79-3.88). By contrast, no association between UGT1A1*6 and grade 3/4 neutropenia was found (Table 3). Among three TS groups, homozygous 3RG/3RG showed least incidence of grade 3/4 late diarrhea (15.8%) and neutropenia (26.3%), although not significantly different from the other two groups ($P > 0.05$). Further subgroup analysis of non-3RG TS genotypes found that patients with TA6/6 were less prone to grade 2-4 late diarrhea than TA7/7 (35.3% *vs* 100.0%, $P = 0.019$). Meanwhile, a trend of lower incidence of grade 3/4 neutropenia was observed in patients with TA6/6, although the association was not statistically significant (29.4% *vs* 50.0%, $P = 0.582$).

Table 3 Association of UDP-glucuronosyltransferase1A1 genotypes with toxicity

Genotypes	Delayed diarrhea						Neutropenia					
	Grades 2-4			Grades 3/4			Grades 3/4			Grade 4		
	n (%)	*P* value[1]	*P* value[2]	*n* (%)	*P* value[1]	*P* value[2]	*n* (%)	*P* value[1]	*P* value[2]	*n* (%)	*P* value[1]	*P* value[2]
UGT1A1*28												
TA 6/6	25 (27.8)	< 0.0005	< 0.0005	14 (15.6)	0.228	0.178	23 (25.6)	0.088	0.055	7 (7.8)	0.043	0.017
TA 6/7	2 (37.5)			6 (18.8)			10 (31.3)			2 (6.3)		
TA 7/7	8 (100)			3 (37.5)			5 (62.5)			3 (37.5)		
UGT1A1*6												
G/G	24 (31.2)	0.564	0.346	10 (13)	0.12	0.057	23 (29.9)	1	0.748	8 (10.4)	0.857	0.473
A/G	19 (39.6)			11 (22.9)			14 (29.2)			4 (8.3)		
A/A	2 (40)			2 (40)			1 (20)			0 (0)		

[1]Fisher's exact test for all genotype; [2]Exact test of Cochran-Armitage trend test across genotypes. Toxicity grade by National Cancer Institute Common Toxicity Criteria version 3.0. A total of 130 patients were assessable for toxicity. Significant differences were found on grades 2-4 delayed diarrhea and grade 4 neutropenia for UGT1A1*28 genotypes. UGT1A1: UDP-glucuronosyltransferase1A1.

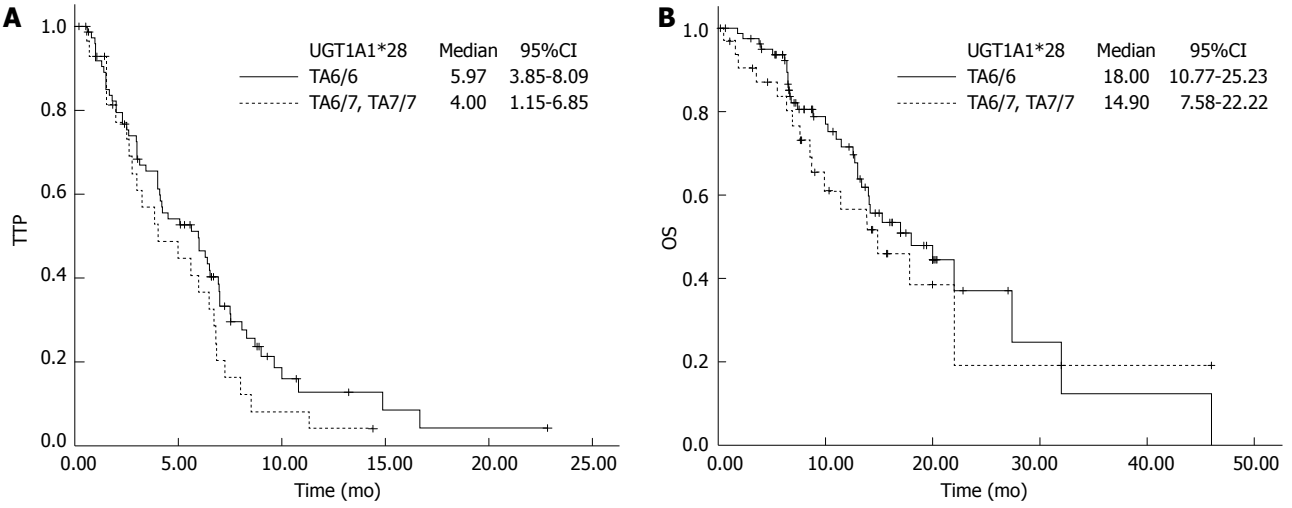

Figure 3 Time to progression and overall survival according to UDP-glucuronosyltransferase1A1*28 genotypes. A: Time to progression (TTP): Patients with TA6/6 were 5.97 (95%CI: 3.85-8.09), patients with TA6/7 and TA7/7 were 4.00 (95%CI: 1.15-6.85), $P = 0.154$; B: Overall survival (OS): Patients with TA6/6 were 18.00 (95%CI: 10.77-25.23), patients with TA6/7 and TA7/7 were 14.90 (95%CI: 7.58-22.22), $P = 0.444$. No significant difference was found between TA6/6 (wild type) and mutated genotypes. UGT1A1: UDP-glucuronosyltransferase1A1.

UGT1A1 polymorphisms correlation with TTP and OS

In 111 patients available for survival outcome analysis, no significant association with TTP and OS was observed with either UGT1A1*28 or UGT1A1*6, although slightly longer TTP and OS were found with UGT1A1*28 (TA6/6) (Figure 3). An additional study of 108 patients on TS revealed that there was no significant association between TS genotype and TTP/OS.

We further examined if the length of treatment duration was related to genotype. Patients with UGT1A1*28 (TA6/6) had a relatively longer treatment time compared with TA6/7 + TA7/7 genotype; near to statistical significance [9.57 ± 6.82 wk (range: 2-32 wk) *vs* 6.76 ± 5.05 wk (range: 1-19 wk), $P = 0.078$]. Although the 3RG homozygous group had longer treatment duration, it failed to display a significant correlation [3RG *vs* non-3RG, 9.03 ± 6.78 wk (range: 1-23 wk) *vs* 8.55 ± 6.16 wk (range: 2-32 wk), $P = 0.895$]. The longest TTP (6.9 mo) and OS (20 mo) were found in the subgroup with TA6/6 and 3RG genotypes, but the differences were still insignificant compared with other subgroups ($P = 0.46$ and 0.37, respectively). Independent variables that might affect OS were tested *via* Cox regression model analysis. These variables were ECOG performance status, age, sex, chemotherapy regimen, UGT1A1 and TS polymorphisms. Only chemotherapy regimen and ECOG were independent prognostic factors affecting OS. Higher ECOG score increased the death risk > 3 times [hazard ratio (HR): 3.325, 95 CI: 1.913-5.777, $P < 0.0005$). Compared with IFL, FOLFIRI showed a 69% decline in death risk (HR: 0.312, 95%CI: 0.132-0.738, $P = 0.008$). For TTP, the Cox model suggested that chemotherapy regimen and sex were independent prognostic factors. Treatment with FOLFIRI lowered the risk of disease progression by 78% (HR: 0.217, 95%CI: 0.104-0.451, $P < 0.0005$). Female sex reduced the progression risk to 60% (HR: 0.608, 95%CI: 0.387-0.968, $P = 0.036$).

Table 4 Association between UDP-glucuronosyltransferase1A1 genotypes and pharmacokinetics parameters

Genotype	AUC CPT-11		AUC SN-38		AUC SN-38G		AUC SN-38G/AUC SN-38 ratio	
	Median	*P* value	Median	*P* value	Median	*P* value	Median	*P* value
UGT1A1*28								
TA6/6	5554.57	0.163[1]	176.40	0.149[1]	582.14	0.988[1]	3.178	0.158[1]
TA6/7	6919.11		213.25		591.86		3.330	
TA7/7	9049.03		390.00		584.63		1.488	
UGT1A1*6								
G/G	5811.73	0.953[2]	189.62	0.320[2]	529.96	0.234[2]	2.924	0.591[2]
G/A	6582.17		231.50		679.17		3.759	
UGT1A1*28+*6								
TA6/6 + G/G	5297.41	0.282[1]	169.62	0.084[1]	529.96	0.386[1]	2.924	0.188[1]
TA6/7 + G/G, TA6/6 + G/A	6649.50		217.87		679.17		3.680	
TA7/7 + G/G TA6/7+ G/A	6213.46		297.89		584.63		1.488	
UGT1A1*28								
TA6/6, TA6/7	6030.13	0.884[2]	191.92	0.067[2]	582.14	1.000[2]	3.177	0.057[2]
TA7/7	9049.03		390.00		584.63		1.488	
UGT1A1*28+*6								
TA6/6 + G/G, TA6/7 + G/G, TA6/6 + G/A	6030.13	0.748[2]	183.37	0.061[2]	582.14	0.915[2]	3.177	0.078[2]
TA7/7 + G/G, TA6/7 + G/A	6213.46		297.89		584.63		1.488	

[1]Kruskal-Wallis test; [2]Mann-Whitney *U* test. 34 patients were assessable for pharmacokinetics parameters. CPT-11: Irinotecan; UGT1A1: UDP-glucuronosyltransferase1A1; AUC: Area under concentration curve.

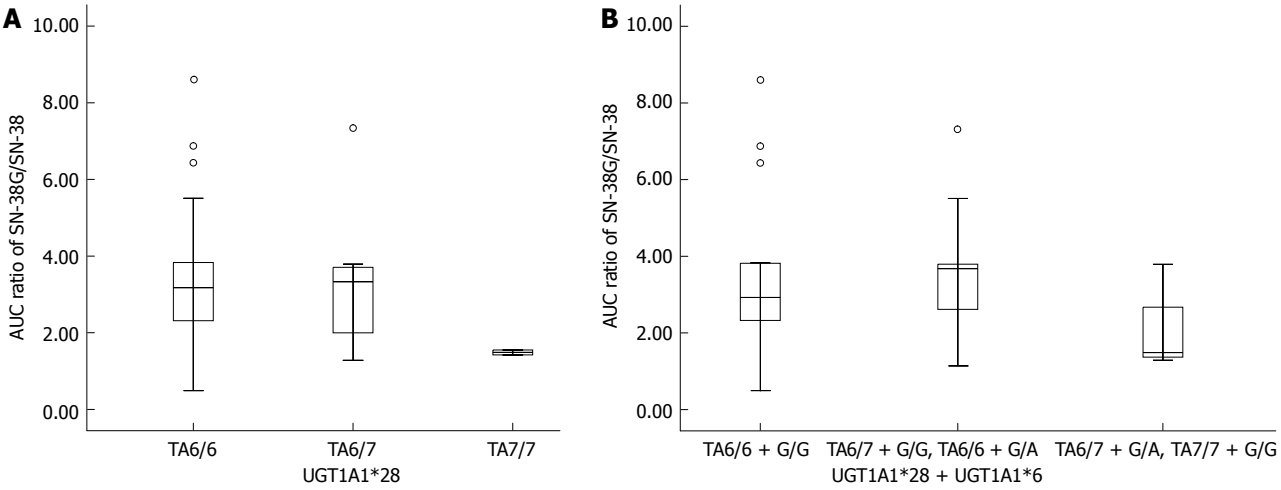

Figure 4 Area under concentration curve ratios of SN-38G/SN-38 comparison according to UDP-glucuronosyltransferase1A1*28 genotypes and UDP-glucuronosyltransferase1A1*28 + *6 group. A: Area under concentration curve (AUC) ratios comparison by UDP-glucuronosyltransferase1A1 (UGT1A1)*28: The AUC ratio of TA7/7 was less than half of the TA6/6 ratio (1.488 *vs* 3.178, *P* = 0.158); B: AUC ratios comparison by UGT1A1*28 + *6 group: Lower AUC ratio was found in the double mutation group (TA6/7 + G/A and TA7/7 + G/G), compared with the other two groups [wild type or single mutation group (1.488 *vs* 3.177, *P* = 0.078)].

*UGT1A1*28 and UGT1A1*6 correlation with pharmacokinetics*

Thirty-four patients were enrolled in a pharmacokinetics study. Among UGT1A1*28 alleles, there were 22 with TA6/6, 10 with TA6/7, and two with TA7/7. Among UGT1A1*6 alleles, there were 25 with G/G and nine with G/A. Among them, two participants were found with a double heterozygous genetic profile; TA6/7 + G/A. The pharmacokinetic parameters showed that the area under concentration curve (AUC) values of irinotecan and SN-38 had an increasing trend among UGT1A1*28 alleles, and TA7/7 appeared to have the highest AUC values, although the AUC values of SN-38G in these three alleles showed no significant difference. Another indicator of SN-38 metabolic rate, the AUC ratio of SN-38G to SN-38 demonstrated that, compared with the other two alleles, the AUC ratio of TA7/7 was lower and close to statistical significance ($P = 0.057$). Due to no A/A allele being identified in UGT1A1*6, we were unable to study the effect of A/A on AUC. The combinations of UGT1A1*28 and UGT1A1*6 were divided into three groups: group 1 with wild type (TA6/6 + G/G); group 2 with one- mutated site variants (TA6/6 + G/A, TA6/7 + G/G); and group 3 with two-mutated site variants (TA6/7 + G/A, TA7/7 + G/A, TA7/7 + G/G). The higher AUC values of irinotecan and SN-38 and lower AUC ratio were found in group 3, and compared with the other two groups, the difference was near to statistical significance ($P = 0.061$ and 0.078, respectively) (Table 4). The plot of AUC ratios (Figure 4) clearly illustrated that

group 3 had lower AUC ratio than the other two groups. Regarding UGT1A1*28, the AUC ratio of TA7/7 was less than half of the TA6/6 ratio (1.488 *vs* 3.178, P = 0.158).

DISCUSSION

Many western studies on the relationship of UGT1A polymorphisms and irinotecan toxicity and response have suggested that UGT1A1*28 is significantly associated with irinotecan-induced toxicity[16-18]. In particular, patients bearing UGT1A1*28 (TA7/7) have a high possibility to experience severe neutropenia and diarrhea. Based on this, a warning is labeled on irinotecan that patients with UGT1A1*28 (TA7/7) should start with a reduced dose of irinotecan, although the details of how to adjust the dose have not been specified[19]. By contrast, research in Asian countries has shown a lower incidence of UGT1A1*28 (TA7/7), while UGT1A1*6 (A/A) is more often found and may replace UGT1A1*28 as a key regulator in UGT1A1 expression[20-22].

Our early studies have found that few carrying UGT1A1*28 (TA7/7) might be the reason that less than expected diarrhea was observed in Chinese patients treated with irinotecan-based chemotherapy. Nevertheless, other UGT1A members, such as UGT1A1*6, UGT1A7*3 and UGT1A9*1 have a trend towards a high incidence of toxicity, and this association is racially different[1]. Although, recent data have implicated that UGT1A7 and UGT1A9 might be useful in predicting irinotecan-related toxicity[7], it might not be applicable for Asian patients because UGT1A1*6 is highly linked with UGT1A7 and UGT1A9 in the Asian population. In addition, Fujita *et al*[4] and Toffoli *et al*[5] have proposed that the homozygous UGTlAl*28 (TA7/7) is a predictive marker for better treatment outcome and longer survival time. The results of our study confirmed that only 6.6% of Chinese patients had UGT1A1*28 (TA7/7), whereas a higher frequency of TA6/6 was identified compared with that in Caucasians (69.9% *vs* 45.2%, P = 0.002). The allele distribution difference might account for lower incidence of severe late diarrhea and grade 3/4 neutropenia in Chinese patients treated with irinotecan. At the same time, we discovered that the association between UGT1A1*6 and grade 3/4 late diarrhea was close to statistically significance. Furthermore, we did not find that treatment efficacy was discounted by either UGT1A1*28 or UGT1A1*6 variants. Besides that, multivariate logistic regression analysis failed to show that UGT1A1/TS polymorphisms and chemotherapy regimen had any effect on clinical response, but indicated that longer treatment time increased response rate by 26%. No OS benefit was obtained in patients with high incidence of toxicity. This could be caused by shorter treatment duration due to drug intolerance.

Several studies have indicated that TS polymorphism is associated with 5-FU toxicity and prognosis. Although the results are inconclusive, it is well accepted that the allele 3RG/3RG is correlated with long OS and low toxicity[23,24]. We introduced TS polymorphism into treatment outcome and toxicity evaluation of irinotecan/5-FU chemotherapy and found that patients carrying UGT1A1*28 (TA6/6) and 3RG/3RG seemed to experience mild toxicity, but relatively low tumor response. Similar to UGT1A1*28, the OS of patients with 3RG/3RG was extended because therapy was well tolerated and its duration was prolonged. However, these observed differences did not reach statistical significance. We conjectured that the differences of treatment response and toxicity between TS genotypes could be narrowed by the synergistic effect of 5-FU combined with irinotecan regimen.

Unfortunately, the combination of two genetic markers was unable to increase the predictive accuracy. In our study, blood samples were used for polymorphism testing. Possibly, examining TS polymorphism and expression level in tumor tissues might yield more convincing data.

The *in vivo* metabolism of irinotecan is complicated. Our data showed that the plasma concentrations of irinotecan and its metabolites varied significantly in each patient. These variations are strongly related to the polymorphisms of many metabolic enzymes and transport proteins. We found that the AUC values of irinotecan and SN-38 were gradually elevated from TA6/6 to TA7/7, whereas the AUC ratios of SN-38G/SN-38 were decreased from TA6/6 to T7/7 (P = 0.057). A similar trend was found for UGT1A1*6. If UGT1A1*28 and UGT1A1*6 were taken together, the AUC ratios of group 1 (wild type), group 2 (one-mutated site) and group 3 (two mutated sites) were shifted from the highest to the lowest. Clearly, both alleles had effects on irinotecan metabolism.

Toffoli *et al*[5] concluded that for Caucasians, UGT1A1*28 not only affected irinotecan-related toxicity, but also changed the drug metabolic rate. On the contrary, reports from Asia suggested that UGT1A1*6 was involved in irinotecan pharmacokinetics and toxicity. Han *et al*[21] discovered that few UGT1A1*28 variants were found in the Korean population, while more UGT1A1*6 variants were observed. They claimed that the role of UGT1A1*28 in predicating irinotecan pharmacokinetics and toxicity could be replaced by UGT1A1*6 for Koreans. The research from Jada *et al*[24] also downplayed the role of UGT1A1*28 in the irinotecan metabolic pathway. However, Minami *et al*[20] contradicted this hypothesis by showing that both UGT1A1*28 and UGT1A1*6 were involved in irinotecan pharmacokinetics. Thus, for the Japanese population, the homozygous (UGT1A1*28/*28 and UGT1A1*6/*6) and the heterozygous (UGT1A1*6/*28) reduced the AUC ratios of SN-38G/SN-38. Our data favored Minami's conclusion that both UGT1A1*28 and UGT1A1*6 participated in glucuronidation.

Derived from the AUC ratios of UGT1A1*28 and UGT1A1*6 variants, we estimated that genetic alterations could inactive the metabolic efficiency of SN-38 by 50%. Therefore we recommend a 50% dose reduction of irinotecan to treat group 3 patients with double-site mutations. Minami *et al*[20] described that the ratio of SN-38 AUC to irinotecan dose was 2.4 in group 3, while the ratio was 1.4

in group 1. Based on their findings, it would be rational to lower the initial dose of irinotecan by 50% for group 3 patients. Another study by Innocenti *et al*[25] investigated the association between the pharmacokinetics of UGT1A1*28 variants and neutropenia. They recommended that a 20% decrease in drug dose should be considered in patients with UGT1A1*28 (TA7/7).

To date, no clinical trial has used genotypes for determining the proper dose for initial irinotecan-based therapy. We recommend a 50% dose reduction of irinotecan for patients with double-site mutations to avoid severe toxicity, and ensure better efficacy with sufficient treatment given. However, accurate dose modification for patients with different *UGT1A1* genotype is difficult. The limitations of our pharmacokinetics study were lack of UGT1A1*6 A/A information and small sample size. A large-scale prospective trial focused on dose modification of irinotecan will be our next work.

ACKNOWLEDGMENTS

We thank Guang-Tao Hao, MD, for his contribution to the PK analysis.

COMMENTS

Background

Irinotecan is a prodrug that is hydrolyzed by carboxylesterase *in vivo* to form an active metabolite SN-38. SN-38 is further conjugated and detoxified by UDP-glucuronosyltransferase (UGT) to yield its β-glucuronide. Remarkable inter-individual variations in the pharmacokinetics and clinical outcomes have been reported. Genetic polymorphisms of UDP-glucuronosyltransferase1A1 (UGT1A1), especially UGT1A1*28 and UGT1A1*6 are important determinants of individual variations in Asian patients. 5-Fluorouracil (5-FU) is a fundamental component of all chemotherapic combinations for treatment of colorectal cancer (CRC). Thymidylate synthetase (TS) is the main intracellular target of fluoropyrimidines. The *TS* gene polymorphisms are known to influence the activity of TS, which are related to 5-FU clinical response. The allele containing the triple repeat (3R) in 5'-UTR of the TS is associated with 3-4-fold translational efficiency compared with the double repeat allele (2R), and a G>C base change in 3R alleles makes the transcriptional activity of the 3R allele as low as that of the 2R allele.

Research frontiers

Current studies have revealed the relationship of UGT1A polymorphisms and irinotecan related toxicity and pharmacokinetics. The polymorphisms of UGT1A1*28 are considered to be important in the Caucasian population, while UGT1A1*6 seems to be more important than UGT1A1*28 in Asian studies. It is still unclear whether UGT1A1 has any influence on treatment efficacy. Three studies tested if UGT1A1 isoforms had any impact on treatment outcome, however, their conclusions were inconsistent. TS polymorphisms are of the most broadly studied genetic variants in CRC. It is accepted that the 3R allele is associated with increased expression levels of TS and poorer outcome in patients treated with 5-FU-based regimens. However, the role of gene polymorphisms of UGT1A1 and TS on clinical response to irinotecan and 5-FU-based treatment, non-diarrhea toxicities and prognosis remains unclear.

Innovations and breakthroughs

Only 6.6% of Chinese patients had UGT1A1*28 (TA7/7), whereas higher frequency of TA6/6 was identified compared with that in Caucasians (69.9% *vs* 45.2%, $P = 0.002$). Mutant variants of UGT1A1*28 and UGT1A1*6 were associated with increased toxicity and decreased SN-38 disposition, but the response rate did not increase accordingly, although this group of patients surely had a trend towards a better response. This probably related to shorter treatment time in patients carrying double-site mutations. Derived from the area under concentration curve ratios of UGT1A1*28 and UGT1A1*6 variants, authors estimated that genetic alterations could inactive the metabolic efficiency of SN-38 by 50%. Therefore, the authors recommend a 50% dose reduction of irinotecan to treat patients with double-site mutations.

Applications

To date, no clinical trial has used genotypes for determining the proper dose for initial irinotecan-based therapy. The authors recommend a 50% dose reduction of irinotecan for patients with double-site mutations to avoid severe toxicity, and ensure better efficacy with sufficient treatment given. However, accurate dose modification for patients with different *UGT1A1* genotypes is difficult. A large-scale prospective trial focusing on dose modification of irinotecan will be next work.

Peer review

Polymorphisms predict response and toxicity in patients with metastatic CRC treated with irinotecan and 5-FU. This study is interesting, and the manuscript is worthy of publication.

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S- Editor Gou SX **L- Editor** Kerr C **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v18.i45.6645

World J Gastroenterol 2012 December 7; 18(45): 6645-6650
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Safety of lamivudine treatment for chronic hepatitis B in early pregnancy

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Received: June 15, 2012 Revised: September 12, 2012
Accepted: September 19, 2012
Published online: December 7, 2012

Abstract

AIM: To evaluate the safety of lamivudine (LAM) treatment for chronic hepatitis B in early pregnancy.

METHODS: A total of 92 pregnant women who received LAM treatment either before pregnancy or in early pregnancy were enrolled in this study. All of the pregnant women volunteered to take lamivudine during pregnancy and were not co-infected with hepatitis C virus, human immunodeficiency virus, cytomegalovirus, or other viruses. All infants received passive-active immunoprophylaxis with 200 IU hepatitis B immunoglobulin and three doses of 10 μg hepatitis B vaccines (0-1-6 mo) according to the guidelines for the prevention and treatment of chronic hepatitis B. Adverse events were observed throughout the entire pregnancy and perinatal period, and the effectiveness of lamivudine treatment for blocking mother-to-infant transmission of hepatitis B virus (HBV) was evaluated. All adverse events in mothers and infants during pregnancy and the perinatal period and the HBV mother-to-infant transmission blocking rate were compared with the literature.

RESULTS: Among the 92 pregnant women, spontaneous abortions occurred in 11 cases, while 3 mothers had a second pregnancy after the initial abortion; 72 mothers delivered 73 live infants, of whom 68 infants were followed up for no less than 6 mo, and 12 mothers were still pregnant. During pregnancy, the main maternal adverse events were vaginitis (12/72, 16.7%), spontaneous abortion (11/95, 11.6%), and gestational diabetes (6/72, 8.3%); only one case had 1-2 degree elevation of the creatine kinase level (195 U/L). During the perinatal period, the main maternal adverse events were premature rupture of the membranes (8/72, 11.1%), preterm delivery (5/72, 6.9%), and meconium staining of the amniotic fluid (4/72, 5.6%). In addition, 2 infants were found to have congenital abnormalities; 1 had a scalp hemangioma that did not change in size until 7 mo, and the other had early cerebral palsy, but with rehabilitation training, the infant's motor functions became totally normal at 2 years of age. The incidence of adverse events among the mothers or abnormalities in the infants was not higher than that of normal mothers or HBV-infected mothers who did not receive lamivudine treatment. In only 2 cases, mother-to-infant transmission blocking failed; the blocking rate was 97.1% (66/68), which was higher than has been previously reported.

CONCLUSION: Lamivudine treatment is safe for chronic HBV-infected pregnant mothers and their fetuses with a gestational age of less than 12 wk or throughout the entire pregnancy.

Key words: Pregnancy; Chronic hepatitis B; Lamivudine; Safety; Hepatitis B virus

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Yi W, Liu M, Cai HD. Safety of lamivudine treatment for chronic hepatitis B in early pregnancy. *World J Gastroenterol* 2012;

18(45): 6645-6650 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i45/6645.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i45.6645

INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a global health problem; about two billion people have a past or present HBV infection, of whom 350 million are chronic HBV carriers. Each year, about one million people die of liver failure, cirrhosis, or primary hepatocellular carcinoma, all of which are associated with HBV infection. Lamivudine (LAM) is the first approved oral nucleoside analog (NA) used to treat HBV infection (100 mg/d), and the appearance of LAM marked a new era in the treatment of chronic HBV infection. However, treatment duration is very long, and some patients may become pregnant during that time. Although some studies have previously reported on the safety of LAM treatment for chronic HBV infection in late pregnancy[1-3], there are very few reports about the safety of LAM treatment in early pregnancy[4-6]. The objective of this study was to evaluate the safety of LAM treatment for chronic hepatitis B in early pregnancy or throughout the entire pregnancy, to provide information about how to block mother-to-infant transmission of HBV in chronic HBV-infected fertile women.

MATERIALS AND METHODS

Patients

All patients were chronic HBV-infected fertile women from our outpatient department who either intended to become pregnant or were already in early pregnancy (with a gestational age of less than 12 wk). Three groups of patients were considered: (1) those who were on NA treatment and could not stop treatment; (2) those who had never taken any NA treatment, with alanine aminotransferase (ALT) > 2 times the upper limit of normal (ULN) for the reference range, HBV DNA > 1×10^5 copies/mL, but failed the traditional liver protection and enzyme reducing treatment; and (3) those who had received NA treatment, but later had virological breakthrough and liver function rebound without LAM drug-resistance. If patients from the above groups planned to become pregnant or had an accidental pregnancy and agreed to LAM treatment after thorough communication and consideration, they were included in this study after providing their written, informed consent.

All participants took screening tests before or in early pregnancy to rule out infection with hepatitis C virus, hepatitis delta virus, human immunodeficiency virus (HIV), syphilis, toxoplasmosis, rubella, cytomegalovirus, and herpes simplex. In this way, these tests helped to exclude any potentially hereditary diseases and any other diseases that needed to be treated. Furthermore, all participants routinely took folic acid before or during early pregnancy to prevent embryonic neural tube defects.

Methods

All participants undertook routine screening tests, and additionally liver function and HBV serology (HBV markers and HBV DNA) were measured every 12 wk. All adverse events that occurred during pregnancy and all neonatal abnormalities were recorded. The rate of blocking vertical transmission of HBV was also determined.

All infants received passive-active immunoprophylaxis with 200 IU hepatitis B immunoglobulin (HBIG) and three doses of 10 μg hepatitis B vaccines (0-1-6 mo) according to the guidelines for the prevention and treatment of chronic hepatitis B[7]. One month later, after all vaccinations (7-8 mo), liver function and HBV serology were measured again to evaluate the blocking rate. All infants underwent routine physical examination, hearing screening, and tests for congenital phenylketonuria and hypothyroidism. All neonatal abnormities were observed for up to 2 years.

Laboratory tests

Liver function and HBV serology were tested in the hospital's clinical laboratory. HBV DNA was detected with an HBV real-time PCR amplification kit from Kehua Biological Company (Shanghai, China), which can detect HBV DNA at levels as low as 500 copies/mL. HBV markers were detected by enzyme-linked immunosorbent assay kits (Abbot Labs, North Chicago, IL, United States) on an ARCHITECT i2000 automatic immunoassay analyzer (Abbott) according to the manufacturer's instructions. Hearing screening was performed with the ECHO-SCREEN from Madsen Company (Germering, Germany). Heel blood was taken on filter paper from the infants after 72 h of breastfeeding, and specimens were sent to the Beijing Neonatal Diseases Screening Center to rule out congenital phenylketonuria and hypothyroidism.

RESULTS

Mothers' basic information

From January 1, 2007 to December 31, 2011, 92 HBV-infected women took LAM treatment before or during early pregnancy. Of these, one mother underwent *in vitro* fertilization with the transfer technique and later delivered twin infants. By the end of 20 wk' gestational age, 11 fetuses aborted, and 3 mothers had a second pregnancy after initial abortion. Ultimately, 72 mothers delivered 73 live infants, of whom 68 infants were followed-up for more than 6 mo, 47 were followed-up for more than 1 year, and 16 were followed-up for more than 2 years. At the end of follow-up, 12 mothers were still pregnant (Figure 1).

Table 1 summarizes the clinical information for the mothers who chose lamivudine treatment before or in early pregnancy, including their demographic characteristics, HBV infection status, and treatment history. All participants were chronic hepatitis B patients, except

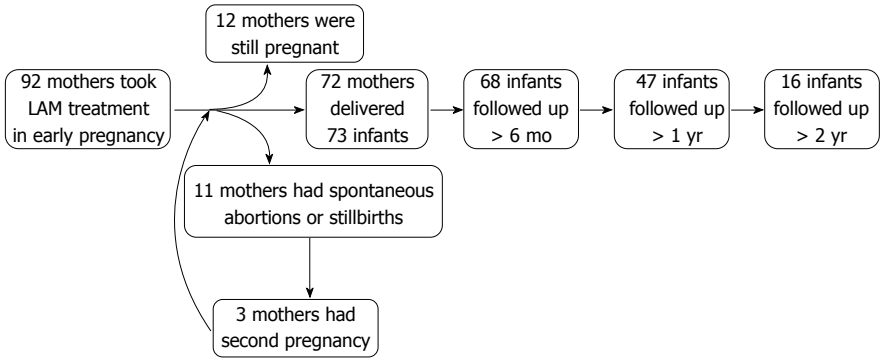

Figure 1 Basic information of mothers and follow-up times for mothers and infants. LAM: Lamivudine.

Table 1 Clinical information of mothers who took lamivudine treatment before or during early pregnancy *n* (%)

	Treated population (*n* = 92)
Average age (yr)	30.5 ± 3.1
Primipara	88 (95.7)
Cirrhosis	2 (2.2)
HBeAg positivity rate	74 (80.4)
HBV DNA > 10^5 copies/mL in early pregnancy	16 (17.4)
Treatment before pregnancy	
Naive	15 (16.3)
LAM	41 (44.6)
ADV	28 (30.4)
LAM→ADV	3 (3.3)
ETV	2 (2.2)
ETV→ADV	1 (1.1)
IFN + LdT	2 (2.2)

LAM: Lamivudine; ADV: Adefovir dipivoxil; ETV: Entecavir; LdT: Telbivudine; IFN: Interferon; HBeAg: Hepatitis B e antigen.

for 2 cases with compensated cirrhosis, and none of the participants were HBV carriers with normal liver function. Most cases had a history of NA treatment before pregnancy; therefore, only 16 cases (17.4%) had HBV DNA > 10^5 copies/mL in early pregnancy. Of these 2 cirrhosis patients, one had refused to take any antiviral treatment despite a long history of abnormal liver function due to her concerns about the safety of NA drugs during pregnancy. However, when she later developed cirrhosis, she started to take LAM treatment and became pregnant; the other patient had concomitant autoimmune hepatitis and was taking ursodesoxycholic acid in addition to adefovir dipivoxil, and was later switched to LAM treatment before pregnancy. In early pregnancy, she ceased ursodesoxycholic acid treatment. In addition, 2 cases had previous peripheral neuropathy or myopathy when they took interferon and telbivudine (LdT) combination treatment; both switched to LAM treatment and later became pregnant.

Adverse events in mothers and infants during pregnancy and the perinatal period

Fetal monitoring during pregnancy: Among the 92 women who took LAM treatment in early pregnancy, there were 95 pregnancies. Before 20 wk' gestational age, 4 cases had a threatened abortion, but symptoms of miscarriage disappeared following aggressive treatment; 11 had developmental arrest or natural abortion, with an abortion rate of 11.6%. Ultimately, 72 mothers delivered 73 live infants; 3 had developmental retardation monitored with ultrasound but development later normalized after improving the mothers' nutritional status and intravenous hydration. There were no other fetal developmental abnormalities reported and no stillbirths.

Monitoring mothers during pregnancy and the perinatal period: All maternal adverse events and laboratory abnormalities during pregnancy and the perinatal period are summarized in Table 2. The top 3 adverse events for mothers during pregnancy were vaginitis (16.7%), gestational diabetes (8.3%), and arrhythmia or abnormal electrocardiogram (5.6%). Only one case had 1-2 degree elevation of the creatine kinase (CK) level (195 U/L), and none of the other adverse events could be associated with LAM treatment. Abnormal ALT levels (≥ 2 × ULN) occurred in 16 cases (22.2%). Of these, 15 cases were in naïve patients without previous antiviral treatment who took LAM treatment only due to abnormal liver function in early pregnancy. The other case stopped LAM treatment before pregnancy and later developed severe hepatitis in early pregnancy, after which LAM was restarted, and her ALT level soon became normal, with her HBV DNA below 500 copies/mL.

However, another patient who took LAM treatment in early pregnancy decreased her HBV DNA by only 1.32 log in 12 wk, after which she was switched to LdT treatment from week 26. In addition, 6 cases had HBV DNA breakthrough with a LAM resistance rate of 8.3%; of these, 3 cases had HBV DNA rebound to above 10^6 copies/mL, and ADV was added from week 28. For the other 3 cases, ADV was added postpartum. The top 3 adverse events for mothers during the perinatal period were premature rupture of the membranes (11.1%), preterm delivery (6.9%), and meconium staining of the amniotic fluid (5.6%). There were no cases of postpartum hemorrhage or perinatal mortality. In addition, HBV DNA was monitored in mothers antepartum, and 63 cases (87.5%) had HBV DNA below 500 copies/mL.

Postpartum infant monitoring

Of the 92 mothers who took LAM treatment before or

Table 2 Adverse events and laboratory abnormalities during pregnancy and the perinatal period (n = 72) (%)

	Adverse events	Cases
Pregnancy	Vaginitis	12 (16.7)
	Gestational diabetes	6 (8.3)
	Arrhythmia/abnormal ECG	4 (5.6)
	Nausea and vomiting	3 (4.2)
	Common cold	3 (4.2)
	Oligohydramnios	3 (4.2)
	Polyhydramnios	2 (2.8)
	Greater than moderate anemia (Hb < 9 g/L)	2 (2.8)
	Placenta previa	2 (2.8)
	Inguinal hernia	1 (1.4)
	Hypothyroidism	1 (1.4)
	ALT ⩾ 2 × ULN	16 (22.2)
	HBV DNA breakthrough	6 (8.3)
	Elevated total bilirubin	2 (2.8)
	Thrombocytopenia	2 (2.8)
	Elevated CK (1-2 degrees)	1 (1.4)
Perinatal period	Premature rupture of the membranes	8 (11.1)
	Preterm	5 (6.9)
	Meconium staining of the amniotic fluid 2-3 degrees	4 (5.6)
	Antepartum hemorrhage	3 (4.2)
	Placenta accreta	1 (1.4)

ECG: Electrocardiogram; Hb: Hemoglobin; ALT: Alanine aminotransferase; ULN: Upper limit of normal; HBV: Hepatitis B virus; CK: Creatine kinase.

in early pregnancy, 72 delivered 73 live infants. Only 3 infants (4.1%) were low birth weight (birth weight < 2500 g); all the other infants had normal weights. None of the infants had abnormal hearing, congenital phenylketonuria, or hypothyroidism on testing. Of the 73 infants, 68 were followed-up for no less than 6 mo, while 47 were followed-up for more than 1 year, and 16 were followed-up for more than 2 years. Two infants were found to have abnormalities postpartum: 1 had 2 scalp hemangiomas (1.5 cm × 1.5 cm and 1.5 cm × 2.0 cm, respectively), which did not change in size until 7 mo, when the parents planned to arrange surgery for the infant; the other infant had early cerebral palsy at 8 mo, but with rehabilitation training, the infant's motor functions became totally normal at 2 years of age. Sixteen babies were followed up for 2 to 4 years, and showed no signs of abnormal intelligence or growth.

Monitoring the mother-to-infant transmission blocking rate

Of the 73 live infants, 68 completed all of the examinations required for evaluating if there was mother-to-infant transmission. Vertical transmission was successfully blocked in 66 infants, for a blocking rate of 97.1%. One mother took LAM every other day by herself because she was concerned about LAM's influence on the infant. She later developed virological breakthrough with HBV DNA increasing to 6.38 × 10^7 copies/mL. The other mother, who had HBV DNA of 3.6 × 10^3 copies/mL antepartum, experienced HBV-S mutation during treatment, resulting in blocking failure (the infant had HBsAg(-), HBV DNA(+) and abnormal ALT levels at both 7 mo and 1 year).

DISCUSSION

HBV infection is a serious public health problem worldwide. According to WHO statistics, about 5% of mothers are chronic HBV carriers[8], and the hepatitis B surface antigen positivity rate among fertile women in some high epidemic areas, such as Africa and South Asia, can be as high as 9.2%-15.5%[9-11]. About one-third of HBV-infected women enter into the immune clearance phase before or during pregnancy, with a high HBV DNA load and abnormal ALT levels. They are not only faced with a high risk of mother-to-infant transmission, but they also have an increased chance of hepatic disease exacerbation during pregnancy, threatening the safety of both mother and infant[12-14]. It is currently not recommended for chronic HBV-infected fertile women who are not pregnant to take interferon or NA treatment, in line with the guidelines for the prevention and treatment of chronic hepatitis B[7]. The antiviral efficacy of interferon is limited, and several side effects hinder its use, resulting in treatment failure. NA treatment alone results in only 20% of hepatitis B e antigen (HBeAg)-positive patients achieving HBeAg seroconversion, with only 12% able to stop treatment with a sustained virological response[15]. Therefore, many chronic HBV-infected fertile women become pregnant during treatment.

It is very dangerous for pregnant women to stop taking antiviral treatment during pregnancy when they have not met the withdrawal standard, as it may exacerbate liver disease, threatening the safety of both mother and infant. However, the safety of infants exposed to antiviral drugs in utero throughout the entire pregnancy is of particular concern, especially in early pregnancy, which is vital for fetal development. Although LAM is already approved as an optional antiviral drug for use in pregnancy, and several studies have reported its safety in late pregnancy[1-3], all of the studies regarding its safety before or during early pregnancy come from HIV-infected pregnant women[16]. Furthermore, most of these patients received combination therapy. So far, there have been very few reports about the safety of LAM treatment in chronic HBV-infected women before or during early pregnancy, and systematic observations have been scarce[4-6,17].

The present study showed that the abortion rate for HBV-infected women who took LAM treatment in early pregnancy was 11.6%, which was not higher than that of non HBV-infected mothers or HBV-infected mothers according to previous reports (11%-16% and 16.7%-21.9%, respectively)[6,18,19]. Overall, 72 mothers delivered 73 live infants; none were stillborn. Three fetuses had developmental retardation during pregnancy monitoring, although their development later normalized after treatment; no other fetal developmental abnormalities were reported. Only 4.1% of infants had low birth weight, which was similar to that of women without or with HBV infection according to previous reports (2.7%-7.8% and 5.0%-10.4%, respectively)[20]. None of the infants had abnormal hearing, congenital phenylke-

tonuria, or hypothyroidism on testing. Two infants were found to have abnormalities (scalp hemangiomas and early cerebral palsy), with a congenital abnormity rate of 2.7%, which is similar to the data from HIV-infected women (3.1%) who took antiretroviral treatment (including LAM) in early pregnancy from 1989 to 2011[16]. According to the literature, the congenital abnormality rates for infants born to mothers without or with HBV infection were 5.1%-6.3% and 7.2%-10%, respectively[6,20,21]. The present report suggests that it is safe for fetuses to be exposed to LAM in utero for the entire pregnancy or in early pregnancy, and it does not affect fertilization or fetal development or result in congenital abnormities. Neither does it affect postnatal development.

The most common adverse event for mothers during pregnancy and the perinatal period was vaginitis (16.7%), among which 7 were vulvovaginal candidiasis (9.7%) and 5 were bacterial vaginosis. However, vaginitis is a common genital infectious disease in pregnant and nonpregnant women and the incidence of vaginitis in our study group was similar to that reported in the literature; it has been reported that the detection rate of candidal vaginitis in pregnant women is about 10%[22,23], and the incidence of bacterial vaginosis among Asian women is 6.1%[24]. Other adverse events included gestational diabetes, gestational hypertension, nausea and vomiting of pregnancy, oligohydramnios, polyhydramnios, placenta previa, anemia, pre-eclampsia, premature rupture of the membranes, and preterm delivery. The incidence of the above adverse events was not higher than that of mothers without or with HBV infection according to previous reports[25-29]. Only one patient had 1-2 degree elevation of serum CK levels, and none of the other adverse events could be associated with LAM treatment. These results suggest that it is safe for HBV-infected pregnant women to take LAM treatment in early pregnancy or throughout the entire pregnancy.

Pregnancy can not only increase the burden on the liver, but it can also increase adrenal cortical hormone levels, boosting HBV replication and activation of hepatitis B. Therefore, for HBV-infected women, the average increase in the HBV DNA level was 0.4 log in late pregnancy or postpartum, and 25% of HBeAg(-) pregnant women had an increase of HBV DNA > 1 log, accompanied by elevation of ALT levels in late pregnancy or postpartum[13,29]. The incidence of severe hepatitis during the perinatal period (in late pregnancy and one month postpartum) was much higher than in nonpregnant women[12-14,30]. In the present study, all mothers without previous antiviral treatment maintained normal ALT levels throughout the entire pregnancy, and none of them had severe hepatitis. Fifteen mothers who took NA treatment previously had abnormal ALT levels in early pregnancy, but ALT normalized after LAM treatment, and the pregnancy continued. Interestingly, one mother stopped LAM treatment before pregnancy and later developed severe hepatitis in early pregnancy. However, all symptoms later disappeared, liver function normalized and the HBV DNA load became undetectable when she restarted LAM. In addition, 87.5% of patients had HBV DNA below 500 copies/mL antepartum, and the LAM resistance rate was only 8.3% during pregnancy. This suggests that LAM can effectively suppress HBV DNA replication in HBV-infected pregnant women, maintain normal liver function, and lower the incidence of hepatic disease during pregnancy, improving the prognosis of HBV-infected pregnant women.

According to previous reports, for infants born to HBeAg-positive mothers, even after they received passive-active immunoprophylaxis with HBIG and three doses of hepatitis B vaccines, vertical transmission was not blocked in 7%-16.3%[31]. For mothers with an increased HBV DNA load, vertical transmission was not blocked in as many as 23.4%-32% of infants[32]. In the present study, 80.4% of mothers was HBeAg-positive, and all of them had a high HBV DNA load before LAM treatment. However, 63 (87.5%) mothers had HBV DNA below 500 copies/mL antepartum, and the blocking rate was 97.1%; blocking failed in only 2 cases. The first mother had poor compliance, resulting in HBV DNA breakthrough, while the other had an HBV-S mutation during treatment, resulting in failure of vaccine immunization. These results suggest that LAM treatment could increase the vertical transmission blocking rate in HBeAg-positive mothers.

This study suggests that it is safe and effective for chronic HBV-infected pregnant women to take LAM treatment in early pregnancy. Treatment does not increase complications or adverse events for mothers during pregnancy or the perinatal period, has no effect on fertilization or embryonic development, and does not increase the incidence of congenital abnormalities in infants. Furthermore, it increases the blocking rate of mother-to-infant transmission. In conclusion, the benefits of taking LAM treatment in early pregnancy outweigh the risks. However, the sample size of this study was small, and the follow-up time was limited; both need to be optimized in later studies to provide greater insight.

COMMENTS

Background

Chronic hepatitis B virus (HBV) infection is prevalent throughout the world; 2 billion people have been or are infected with HBV worldwide, with 350 million people having chronic infections. Each year, more than 1 million people die of HBV-related liver failure, cirrhosis, or primary hepatic carcinoma. Interferon and nucleos(t)ide analogs (NAs) are available for the antiviral treatment of hepatitis B. However, the efficacy of interferon is suboptimal, and interferon is associated with scores of adverse effects, so that many patients fail or cannot tolerate treatment. Treatment with NAs requires long-term persistence, as in the case of human immunodeficiency virus; stopping the medicine frequently leads to relapse of the disease. As women undergoing treatment cannot halt the medicine at will, pregnancy often occurs during the on-treatment period. However, the safety profile of NAs has not been verified. The goal of this study was to observe the safety profile of lamivudine for the mother and fetus throughout the entire pregnancy.

Research frontiers

In recent years, with many studies evaluating the efficacy and safety profile, the administration of lamivudine and other NAs during the third trimester to block HBV transmission has been a hot topic. However, studies regarding lamivudine

treatment during the first trimester or the entire period of pregnancy are scarce.

Innovations and breakthroughs

This study observed adverse events throughout the entire period of pregnancy in women taking lamivudine, including abortion, ectopic pregnancy, and complications related to pregnancy. The study also observed intrauterine deformations of the fetus and abnormalities after birth in detail and maintained long-term follow-up of some neonates.

Applications

The study provides preliminary evidence of the safety profile for pregnant women taking lamivudine. It also sets an example for further studies exploring the safety profile of NAs in pregnant women.

Peer review

The manuscript describes safety of lamivudine treatment in early pregnancy. They compared maternal and infant abnormality, HBV DNA level and mutations, and blocking rates of mother-to infant transmission between cases with and without lamivudine treatment. They concluded that lamivudine treatment in early treatment is safe. The study is important, because patients with HBV infection often show elevation of HBV DNA levels and may have alanine aminotransferase flare.

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S- Editor Gou SX **L- Editor** A **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v18.i45.6651

World J Gastroenterol 2012 December 7; 18(45): 6651-6656
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Overexpression of lysine specific demethylase 1 predicts worse prognosis in primary hepatocellular carcinoma patients

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Received: June 23, 2012 Revised: August 10, 2012
Accepted: August 25, 2012
Published online: December 7, 2012

Abstract

AIM: To investigate the clinicopathological features and prognostic value of lysine specific demethylase 1 (LSD1) in hepatocellular carcinoma (HCC).

METHODS: We examined LSD1 expression in 60 paired liver cancer tissues and adjacent noncancerous tissues by quantitative real time polymerase chain reaction (qRT-PCR) and Western blotting. In addition, we analyzed LSD1 expression in 198 HCC samples by immunohistochemistry. The relationship between LSD1 expression, clinicopathological features and patient survival was investigated.

RESULTS: Immunohistochemistry, Western blotting, and qRT-PCR consistently confirmed LSD1 overexpression in HCC tissues compared to adjacent non-neoplastic tissues ($P < 0.01$). Additionally, immunostaining showed more LSD1-positive cells in the higher tumor stage (T3-4) and tumor grade (G3) than in the lower tumor stage (T1-2, $P < 0.001$) and tumor grade (G1-2, $P < 0.001$), respectively. Moreover, HCC patients with high LSD1 expression had significantly lower 5-year overall survival rates ($P < 0.001$) and lower 5-year disease-free survival rates ($P < 0.001$), respectively. A Cox proportional hazards model further demonstrated that LSD1 over-expression was an independent predictor of poor prognosis for both 5-year disease-free survival [hazards ratio (HR) = 1.426, 95%CI: 0.672-2.146, $P < 0.001$] and 5-year overall survival (HR = 2.456, 95%CI: 1.234-3.932, $P < 0.001$) in HCC.

CONCLUSION: Our data suggest for the first time that the overexpression of LSD1 protein in HCC tissues indicates tumor progression and predicts poor prognosis.

Key words: Hepatocellular carcinoma; Lysine specific demethylase 1; Tumor progression; Prognosis

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, especially in Asia[1]. In China, HCC ranks behind gastric and esophageal cancer with the third highest mortality rate among all malignant carcinomas, leads to approximately 110 000 deaths every year, and accounts for 45% of total HCC deaths worldwide[2]. Multiple risk factors have been associated with the initiation and development of HCC; these include chronic infection with hepatitis B, C or D virus, aflatoxin, alcohol abuse, hereditary metabolic liver diseases, and diabetes mellitus[1,3]. Like most other cancers, hepatocarcinogenesis is a multistep process that involves multiple genetic alterations that may activate oncogenes and/or inactivate tumor suppressor genes, ultimately leading to the malignant transformation of hepatocytes.

Histone demethylase lysine specific demethylase 1 (LSD1), the first histone demethylase that was discovered as a nuclear homolog of amine oxidases, removes the methyl groups from mono- and dimethylated lysine (Lys) 4 of histone H3 (H3K4me1/2) and Lys9 of histone H3 (H3K9me1/2). LSD1 is essential for mammalian development and is involved in many biological processes, including cell-type differentiation, gene activation and gene repression[4]. A recent study indicated that LSD1 might promote cell phase transition (deficiency in LSD1 led to partial cell cycle arrest in G2/M) and cell proliferation, suggesting that its over-expression might promote tumorigenesis[5]. The expression of LSD1 has been associated with tumor recurrence during therapy in various cancers, further implicating LSD-1 as a tumor promoter[6-8]. A tissue cDNA microarray analysis also demonstrated the presence of LSD1 transactivation in lung and colorectal carcinomas[7]. LSD1 knockdown with small interfering (si) RNAs resulted in the suppression of proliferation of various bladder and lung cancer cell lines[7]. To the best of our knowledge, there is little available data regarding the involvement of *LSD1* genes in hepatic tumorigenesis. In this study, we investigated LSD1 expression in HCC and its correlation with the clinicopathological features of patients with HCC, including patient survival.

MATERIALS AND METHODS

Patients and tissue samples

The study was approved by the Research Ethics Committee of Xinhua Hospital, which is affiliated to Shanghai Jiaotong University School of Medicine, Shanghai, China. Informed consent was obtained from all of the patients. All specimens were handled and made anonymous according to accepted ethical and legal standards.

A total of 198 patients who presented with primary HCC and later underwent curative liver resection at Xinhua Hospital affiliated to Shanghai Jiaotong University School of Medicine, Shanghai, China, were included in this retrospective study. The tissue samples used in this study were retrieved from the tissue bank of the Department of Pathology in the Xinhua Hospital affiliated with Shanghai Jiaotong University School of Medicine. The patients had been diagnosed with HCC between 2001 and 2006. None of the patients recruited in this study had undergone preoperative chemotherapy or radiotherapy. HCC diagnosis was based on World Health Organization criteria. Tumor differentiation was defined according to the Edmondson grading system. Liver function was assessed using the Child-Pugh scoring system. Tumor staging was determined according to the sixth edition of the tumor-node-metastasis (TNM) classification of the International Union against Cancer. The clinicopathological features of 198 patients are summarized in Table 1. In addition, 60 self-pairs of HCC specimens (10 TNM stage Ⅰ, 16 TNM stage Ⅱ, 24 TNM stage Ⅲ, and 10 TNM stage Ⅳ) and adjacent non-neoplastic liver tissues were snap frozen in liquid nitrogen and stored at -80 ℃ following surgery for quantitative real time polymerase chain reaction (qRT-PCR) assay and western blot analysis. The median follow-up period was 8.6 years. Postoperative surveillance included routine clinical and laboratory examinations every third month, computed tomography scans of the abdomen, and radiographs of the chest every third month. After 5 years, the examination interval was extended to 12 mo.

Table 1 Clinicopathological features and the expression of lysine specific demethylase 1 in 198 hepatocellular carcinoma patients

Characteristics	*n*	LSD1 (%)		*P* value
		High expression	Low expression	
Gender				
Male	101	58	43	
Female	97	54	43	NS
Age (yr)				
⩾ 50	112	61	51	
< 50	86	51	35	NS
Tumor stage				
T1	39	5	34	
T2	42	21	21	
T3	76	47	29	
T4	41	39	2	< 0.001
Tumor grade				
G1	45	9	34	
G2	114	63	51	
G3	39	38	1	< 0.001
Growth pattern				
Trabecular	147	84	63	
Nontrabecular	51	28	23	NS
Cirrhosis				
Yes	151	87	64	
No	47	25	22	NS
Underlying liver disease				
Alcoholic	21	15	6	
Hepatitis B	136	74	62	
Hepatitis C	30	17	13	
Unknown	11	6	5	NS

LSD1: Lysine specific demethylase 1; NS: Not significant.

Immunohistochemistry analysis

Immunohistochemical staining was carried out follow-

ing the protocol of our previous study[9-11]. The primary antibody against LSD1 was a rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., United States) at a dilution of 1:50. The specificity of the primary antibody has been validated by the previous studies of Müller *et al*[12] and Lü *et al*[13]. The secondary antibody for the detection of primary antibody was anti-rabbit immunoglobulin G (Sigma, St. Louis, MO, United States). The negative controls were processed in a similar manner with phosphate-buffered saline instead of primary antibody. Further, positive LSD1 expression, as confirmed by western blotting, was used as a positive control for immunostaining. Following hematoxylin counterstaining, immunostaining was scored by two independent experienced pathologists who were blinded to the clinicopathological parameters and clinical outcomes of the patients. The scores of the two pathologists were compared, and any discrepant scores were re-examined for staining by both pathologists until a consensus score was obtained. The number of cells that stained positive for nuclear LSD1 in ten representative microscopic fields was counted, and the percentage of positive cells was calculated. The percentages of cells that were immunoreactive were converted to scores as follows: 0 (0%), 1 (1%-10%), 2 (11%-50%) and 3 (> 50%). Staining intensity was visually scored and stratified as follows: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). A final score was obtained for each case by multiplying the percentage score and the intensity score. Multiplied scores exceeding 5 (median of total scores for LSD1) were considered to indicate low levels of LSD1 expression, while all other scores were considered to indicate high levels of LSD1 expression.

Western blotting

The Western blotting protocol and semiquantitative analysis were carried out following the protocol of Xu *et al*[14]. LSD1 antibody (rabbit polyclonal antibody, dilution 1:50, Santa Cruz Biotechnology, Inc., United States) was used, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (CW0266, dilution 1:1000, CoWin Biotech) was used as an internal control.

Quantitative RT-PCR

To measure the mRNA expression levels of LSD1, total RNA was extracted from frozen liver tissues using TriZol reagent (Invitrogen) according to the manufacturer's instructions. The extraction was followed by RT-PCR using the TransStart Green qPCR SuperMix (TransGen Biotech). The primer sequences for LSD1 amplification were 5'-CGAACGCACATCAAGACGA-3' for the forward primer and 5'-AGGTGAAGGTGGAGTAGAGGC-3' for the reverse primer. The transcription of GAPDH was used as an internal control for normalization. LSD1 expression levels were calculated relative to GAPDH using the delta-delta computed tomography method[15].

Statistical analysis

SPSS version 13.0 for Windows (SPSS Inc, IL, United States) and SAS 9.1 (SAS Institute, Cary, NC) were used for statistical analysis. A Fisher's exact test and χ^2 test were performed to assess associations between LSD1 expression and clinicopathological parameters. A Kaplan-Meier method was used for survival analysis, and differences in survival were estimated using the log-rank test. A multivariate survival analysis was performed for all parameters that were significant in the univariate analyses using a Cox regression model. Differences were considered statistically significant when P was less than 0.05.

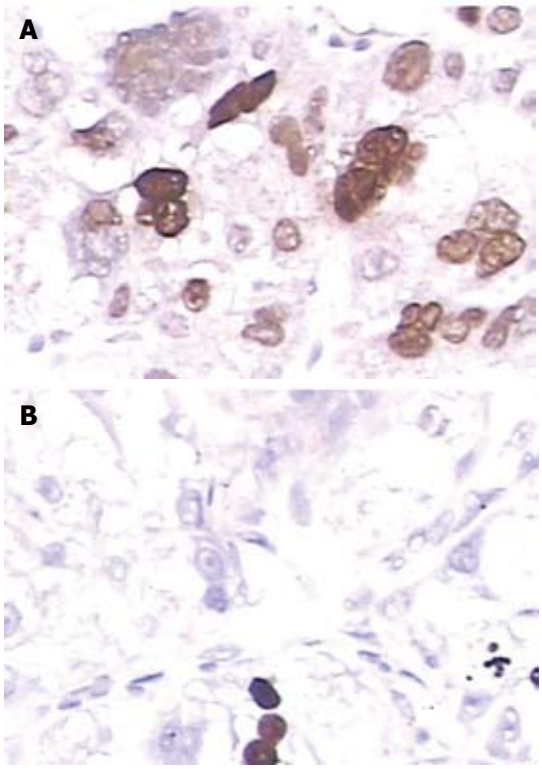

Figure 1 Lysine specific demethylase 1 expression in hepatocellular carcinoma and adjacent non-neoplastic liver tissues. A: High expression of lysine specific demethylase 1 (LSD1) in hepatocellular carcinoma (HCC) samples; B: Low expression of LSD1 in HCC samples (original magnification, 200×).

RESULTS

Expression of LSD1 protein and mRNA in HCC

Immunohistochemical analysis revealed that LSD1 staining was mainly localized to the nucleus of noncancerous and malignant epithelial cells (Figure 1). In addition, we found that 112 (56.6%) of 198 HCC tissues had high LSD1 expression, and 86 (43.4%) of 198 HCC tissues had low LSD1 expression. Additionally, 52 (26.3%) of 198 adjacent non-neoplastic liver tissues had high LSD1 expression, and 146 (73.7%) of 198 adjacent nonneoplastic liver tissues had low LSD1 expression. Thus, the LSD1 immunostaining in HCC tissues was significantly higher than the staining in the adjacent non-neoplastic liver tissues ($P < 0.01$). To confirm LSD1 protein expression by an independent method, Western blotting analysis was performed using 60 self-pairs of HCC and adjacent

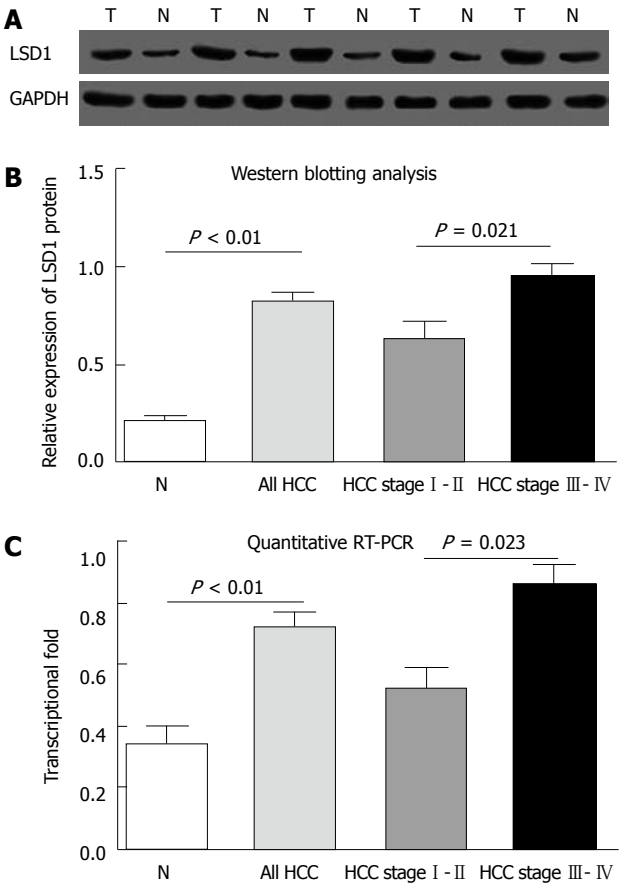

Figure 2 Increased lysine specific demethylase 1 protein and mRNA levels in hepatocellular carcinoma with different tumor node metastasis stages and adjacent non-neoplastic liver tissues. A: Representative Western blotting of lysine specific demethylase 1 (LSD1) protein levels in hepatocellular carcinoma (HCC) tissues (T) and adjacent non-neoplastic liver tissues (N); B: Semiquantitative Western blotting showed that the expression levels of LSD1 protein were significantly higher than those in adjacent non-neoplastic liver tissues ($P < 0.01$). Additionally, the expression levels of LSD1 protein increased with ascending tumor node metastasis (TNM) stages. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. *P* values, mean and SD were given (*t* test); C: Quantitative reverse transcription-polymerase chain reaction (RT-PCR) assay showed significantly increased LSD1 mRNA levels in HCC tissues compared with adjacent non-neoplastic liver tissues ($P < 0.01$). Additionally, the expression levels of LSD1 mRNA were increased with ascending tumor TNM stages. GAPDH was used as the internal control. *P* values, mean and SD were given (Mann-Whitney test).

non-neoplastic liver tissues. Overexpression of LSD1 protein in HCC tissues was compared with adjacent non-neoplastic liver tissues ($P < 0.01$, Figure 2A and B) using this method, and significantly increased LSD1 mRNA levels were detected by qRT-PCR ($P < 0.01$, Figure 2C). The expression levels of LSD1 protein and mRNA in high stage (Ⅲ-Ⅳ) HCC tissues were both significantly higher than the levels of LSD1 protein and mRNA in low stage (Ⅰ-Ⅱ) HCC tissues (for protein: $P = 0.021$; for mRNA: $P = 0.023$; Figure 2B and C).

Association of LSD1 expression with the clinicopathological features of HCC

To evaluate whether LSD1 protein expression was associated with clinicopathological features of patients with

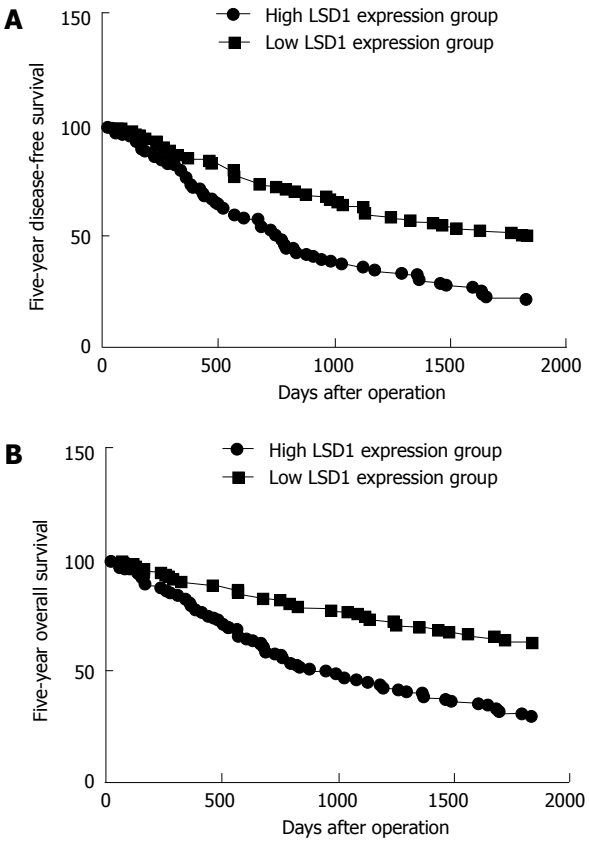

Figure 3 Kaplan-Meier survival curves for lysine specific demethylase 1 expression in hepatocellular carcinoma patients. A: The hepatocellular carcinoma patients with high lysine specific demethylase 1 (LSD1) expression showed significantly shorter disease-free survival ($P < 0.001$); B: Overall survival ($P < 0.001$) rates than those with low LSD1 expression.

HCC, we correlated immunohistochemical LSD1 staining results with T stage, tumor grade, presence of cirrhosis, underlying liver disease, including alcohol abuse, viral hepatitis B and C, sex and age (Table 1). We found more LSD1 positive cells in tissues with higher tumor stages (T3-4) and higher tumor grades (G3) than in the lower tumor stages (T1-2, $P < 0.001$) and tumor grades (G1-2, $P < 0.001$), respectively.

Prognostic values of LSD1 expression in HCC

The 5-year disease-free survival in the group with high LSD1 expression was significantly poorer than the disease-free survival in the group with low LSD1 expression ($P < 0.001$, log-rank test; Figure 3A). A Kaplan-Meier plot of 5-year overall survival curves stratified by LSD1 expression is shown in Figure 3B. There was a significant relationship between LSD1 expression and 5-year overall survival ($P < 0.001$, log-rank test; Figure 3B). In a multivariate Cox model that included tumor size, tumor stage, tumor grading, presence of cirrhosis, gender, age, and LSD1 staining, we found that LSD1 expression independently indicated poor prognosis for both 5-year disease-free survival [hazards ratio (HR) = 1.426, 95%CI: 0.672-2.146, $P < 0.001$; Table 2] and 5-year overall survival (HR = 2.456, 95%CI: 1.234-3.932, $P < 0.001$; Table

Table 2 Multivariate survival analysis of five-year overall and disease-free survival in 198 patients with hepatocellular carcinoma

Parameter	Five-year overall survival			Five-year disease-free survival		
	P value	HR	95%CI	*P* value	HR	95%CI
Age	0.775	0.867	0.463-1.452	0.714	1.174	0.883-1.853
Gender	0.456	1.121	0.569-1.867	0.634	1.126	0.684-1.846
Tumor stage	< 0.001	1.634	1.142-2.537	< 0.001	1.423	0.784-2.161
Tumor grade	< 0.001	1.154	0.647-1.893	< 0.001	1.023	0.456-1.638
Presence of cirrhosis	0.542	1.143	0.647-1.784	0.427	1.321	0.824-1.917
LSD1 expression	< 0.001	2.456	1.234-3.932	< 0.001	1.426	0.672-2.146

LSD1: Lysine specific demethylase 1; HR: Hazards ratio.

2) in patients with HCC.

DISCUSSION

Genetic alterations are a hallmark of human cancer. In recent years, the field of cancer genomics has made significant advances in the area of cancer-associated genetic lesion identification. Furthermore, the importance of epigenetic changes that occur during HCC development has also been recognized[16]. Epigenetic changes can take the form of DNA methylation or histone modification[17]. Histone modifications in the form of selective acetylation, phosphorylation, and methylation serve as switches that alter chromatin structure, allowing posttranscriptional activation or repression of downstream proteins[18]. Understanding these epigenetic changes will lead to the identification of novel cancer-related genes that may represent attractive targets for cancer treatment and provide new insights into the biology of hepatic cancers. Thus, an integrative approach to hepatic cancer research that combines epidemiological, genetic and epigenetic information has emerged as an important paradigm for cancer therapy[19]. The methylation status of histone methyltransferases and histone demethylases plays a pivotal role in the regulation of gene expression[20]. LSD1, the first histone demethylase that was discovered as a nuclear homolog of amine oxidases, removes methyl groups from mono- and dimethylated H3K4me1/2 and H3K9me1/2[21].

Epigenetic changes in LSD1 have been shown to play a key role in carcinogenesis[22]. LSD1 can prevent the accumulation of the dimethyl groups of p53, repressing p53-mediated transcriptional up-regulation, preventing apoptosis, and contributing to human carcinogenesis *via* a chromatin modification mechanism. To date, a few studies have indicated that LSD1 may promote cell phase transition (deficiency in LSD1 led to partial cell cycle arrest in G2/M) and cell proliferation, suggesting that LSD1 over-expression might promote tumorigenesis[5]. The expression of LSD1 has been associated with tumor recurrence during therapy in various cancers, further implicating LSD-1 as a tumor promoter[6-8]. Tissue cDNA microarray analysis also revealed LSD1 transactivation in lung and colorectal carcinomas[7]. Knocking down LSD1 with small interfering RNAs resulted in suppression of proliferation of various bladder and lung cancer cell lines[7]. However, the association between LSD1 and the survival of HCC patients was not well defined. In this study, we investigated the associations between LSD1 expression levels and clinical features of HCC patients.

In order to demonstrate that the epigenetic changes were associated with genetic changes in lung cancer, we first investigated the expression of LSD1 in HCC clinical samples. Previous studies have demonstrated that LSD1 protein and mRNA levels could act as biomarkers for the identification of patients with more aggressive breast cancer, prostate cancer, lung cancer and neuroblastoma[23-26]. In our study, we detected LSD1 by immunohistochemistry analysis, Western blotting, and qRT-PCR. Our results showed that LSD1 immunoreactivity was significantly increased in HCC compared with adjacent non-neoplastic liver tissue in a substantial proportion of cases. The overexpression of LSD1 was observed in tumor tissues with higher tumor stage and higher tumor grade. Additionally, our investigation revealed that high LSD1 expression is associated with a significant trend toward both poorer disease-free survival and poorer overall survival. Our study further confirms that high LSD1 expression independently predicts a higher risk of disease relapse or death after multivariate adjustment for other prognostic factors. These observations support the hypothesis that LSD1 may function as an oncogene in HCC and suggest that LSD1 may play an important role in the tumorigenesis of HCC. However, the role of LSD1 in HCC remains to be elucidated. Our data may offer new insight into LSD1 as a potentially important contributor to the progression of HCC and as a new prognostic factor for HCC. As the 198 cases of the present study were all obtained from the Chinese population, the results reported here should be further confirmed in other populations.

In conclusion, our study suggests that LSD1 is overexpressed in HCC tissues compared with their benign counterparts. To the best of our knowledge, this is the first study evaluating the expression levels of LSD1 mRNA and protein in HCC tissues and the association between these expression levels and clinicopathologic parameters. The most important finding of this study is that LSD1 expression may predict a poorer prognosis for HCC patients after surgery. Further studies are needed to investigate the precise function of LSD1 in the progression of HCC.

COMMENTS

Background

Lysine specific demethylase 1 (LSD1) is essential for mammalian development and is involved in many biological processes, such as cell-type differentiation, gene activation and gene repression. Knocking down LSD1 with small interfering RNAs suppressed the proliferation of various bladder and lung cancer cell lines. To be known, little data has been generated with regard to the involvement of *LSD1* genes in hepatic tumorigenesis. In this study, the authors investigated LSD1 expression in hepatocellular carcinoma (HCC) and its correlation with clinicopathological features, including the survival of patients with HCC.

Research frontiers

The data suggest for the first time that the overexpression of LSD1 protein in HCC tissues may help predict tumor progression and poor prognosis.

Innovations and breakthroughs

The authors examined LSD1 expression in 60 paired liver cancer tissues and adjacent noncancerous tissues by quantitative real time polymerase chain reaction and Western blotting. In addition, authors analyzed LSD1 expression in 198 HCC samples by immunohistochemistry. The relationships between LSD1 expression and both clinicopathological features and patient survival were investigated.

Applications

These findings provide evidence that the overexpression of LSD1 serves as a biomarker for poor prognosis in HCC. Thus, authors speculate that LSD1 may be a potential target of anti-angiogenic therapy for HCC.

Terminology

LSD1, the first histone demethylase that was discovered as a nuclear homolog of amine oxidases, removes the methyl groups from mono- and dimethylated Lysine (Lys) 4 of histone H3 and Lys9 of histone H3.

Peer review

The authors investigated the expression of LSD1 in HCC and determined its correlation with tumor progression and prognosis. The authors claim that the expression levels of LSD1 protein in HCC tissues correlates with tumor progression and prognosis.

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S- Editor Gou SX **L- Editor** A **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v18.i45.6657

World J Gastroenterol 2012 December 7; 18(45): 6657-6668
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

META-ANALYSIS

Meta-analysis of laparoscopic *vs* open liver resection for hepatocellular carcinoma

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Received: March 25, 2012 Revised: September 17, 2012
Accepted: September 22, 2012
Published online: December 7, 2012

Abstract

AIM: To conduct a meta-analysis to determine the safety and efficacy of laparoscopic liver resection (LLR) and open liver resection (OLR) for hepatocellular carcinoma (HCC).

METHODS: PubMed (Medline), EMBASE and Science Citation Index Expanded and Cochrane Central Register of Controlled Trials in the Cochrane Library were searched systematically to identify relevant comparative studies reporting outcomes for both LLR and OLR for HCC between January 1992 and February 2012. Two authors independently assessed the trials for inclusion and extracted the data. Meta-analysis was performed using Review Manager Version 5.0 software (The Cochrane Collaboration, Oxford, United Kingdom). Pooled odds ratios (OR) or weighted mean differences (WMD) with 95%CI were calculated using either fixed effects (Mantel-Haenszel method) or random effects models (DerSimonian and Laird method). Evaluated endpoints were operative outcomes (operation time, intraoperative blood loss, blood transfusion requirement), postoperative outcomes (liver failure, cirrhotic decompensation/ascites, bile leakage, postoperative bleeding, pulmonary complications, intraabdominal abscess, mortality, hospital stay and oncologic outcomes (positive resection margins and tumor recurrence).

RESULTS: Fifteen eligible non-randomized studies were identified, out of which, 9 high-quality studies involving 550 patients were included, with 234 patients in the LLR group and 316 patients in the OLR group. LLR was associated with significantly lower intraoperative blood loss, based on six studies with 333 patients [WMD: -129.48 mL; 95%CI: -224.76-(-34.21) mL; P = 0.008]. Seven studies involving 416 patients were included to assess blood transfusion requirement between the two groups. The LLR group had lower blood transfusion requirement (OR: 0.49; 95%CI: 0.26-0.91; P = 0.02). While analyzing hospital stay, six studies with 333 patients were included. Patients in the LLR group were found to have shorter hospital stay [WMD: -3.19 d; 95%CI: -4.09-(-2.28) d; P < 0.00001] than their OLR counterpart. Seven studies including 416 patients were pooled together to estimate the odds of developing postoperative ascites in the patient groups. The LLR group appeared to have a lower incidence of postoperative ascites (OR: 0.32; 95%CI: 0.16-0.61; P = 0.0006) as compared with OLR patients. Similarly, fewer patients had liver failure in the LLR group than in the OLR group (OR: 0.15; 95%CI: 0.02-0.95; P =

0.04). However, no significant differences were found between the two approaches with regards to operation time [WMD: 4.69 min; 95%CI: -22.62-32 min; *P* = 0.74], bile leakage (OR: 0.55; 95%CI: 0.10-3.12; *P* = 0.50), postoperative bleeding (OR: 0.54; 95%CI: 0.20-1.45; *P* = 0.22), pulmonary complications (OR: 0.43; 95%CI: 0.18-1.04; *P* = 0.06), intra-abdominal abscesses (OR: 0.21; 95%CI: 0.01-4.53; *P* = 0.32), mortality (OR: 0.46; 95%CI: 0.14-1.51; *P* = 0.20), presence of positive resection margins (OR: 0.59; 95%CI: 0.21-1.62; *P* = 0.31) and tumor recurrence (OR: 0.95; 95%CI: 0.62-1.46; *P* = 0.81).

CONCLUSION: LLR appears to be a safe and feasible option for resection of HCC in selected patients based on current evidence. However, further appropriately designed randomized controlled trials should be undertaken to ascertain these findings.

Key words: Hepatocellular carcinoma; Laparoscopy; Open liver resection; Hepatectomy; Meta-analysis

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Xiong JJ, Altaf K, Javed MA, Huang W, Mukherjee R, Mai G, Sutton R, Liu XB, Hu WM. Meta-analysis of laparoscopic *vs* open liver resection for hepatocellular carcinoma. *World J Gastroenterol* 2012; 18(45): 6657-6668 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i45/6657.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i45.6657

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common primary cancer worldwide[1], and the third most common cause of cancer-related deaths with about 600 000 patients dying from the disease annually[2]. The potential treatment options for HCC include: surgical resection[3], liver transplantation[4], chemotherapy and local ablative therapy[5]. Surgery, either through hepatic resection or liver transplantation, is the best hope for a cure, but is not suitable for those patients who also suffer from significant background cirrhosis[5]. Liver transplantation should be considered in any patient with cirrhosis and a small (5 cm or less single nodule or up to three lesions of 3 cm or less) HCC. Hepatic resection, on the other hand, should be considered as a primary therapy in every patient with HCC and a non-cirrhotic liver (including fibrolamellar variant). Resection can also be carried out in highly selected patients with hepatic cirrhosis and well preserved hepatic function (Child-Pugh A) who are unsuitable for liver transplantation[6].

Open liver resection (OLR) has traditionally been accepted as the preferred treatment for resectable HCC in patients with adequate liver reserves[7]. However, most patients with HCC have significant underlying co-morbidities, including liver diseases such as chronic hepatitis and liver cirrhosis, and hence are at very high risk of developing significant postoperative complications. Laparoscopic surgery is considered to be a safe alternative to open surgical intervention in numerous surgical procedures. Since the first successful report of laparoscopic liver wedge resection in 1992[8], improvement in surgical instrumentation and experience in laparoscopic treatment for the majority of surgical gastrointestinal conditions, including benign liver diseases, have led to a growing interest in its application for HCC. Recent studies have suggested that the laparoscopic liver resection (LLR) has a number of advantages such as reduction of postoperative pain, operative morbidity, and length of hospitalization, especially for cirrhotic patients with HCC[9-12]. However, the current literature on LLR for HCC exists in the form of few comparative studies. General application of this approach for treating this disease is still a matter of debate because it is new and data regarding long-term oncologic outcomes (e.g., recurrence) are not robust.

Three published meta-analysis[13-15] have investigated the advantages and disadvantages of the LLR for HCC. These meta-analyses have reported that LLR was associated with decreased blood loss and requirement for blood transfusion, lower overall postoperative morbidity and shorter hospital stay compared with the OLR. In addition, there was no difference between groups in oncologic outcomes such as positive resection margins and tumor recurrence. Since these meta-analysis included a limited number of studies with fewer cases, data reported were not sufficient to derive conclusions with regards to the overall efficacy and safety of LLR. In the interim, several high-quality studies[16-20] with more participants have been published. We have therefore undertaken an analysis of 15 studies including 1105 hepatic resections to provide an update on the efficacy of LLR *vs* OLR for HCC.

MATERIALS AND METHODS

Study selection

PubMed (Medline), EMBASE and Science Citation Index Expanded and Cochrane Central Register of Controlled Trials in the Cochrane Library were searched systematically for all articles published from January 1992 to February 2012 comparing LLR and OLR for HCC. The following medical search headings and keywords were used: "laparoscopy" or "laparoscopic" or "minimally invasive surgery" and "hepatectomy" or "liver resection" or "hepatic resection" and "primary liver carcinoma" or "hepatocellular carcinoma" or "HCC". Only human studies published in English language as full text articles were considered for inclusion. Reference lists of selected articles were also examined to find relevant studies which were not identified during the initial data-

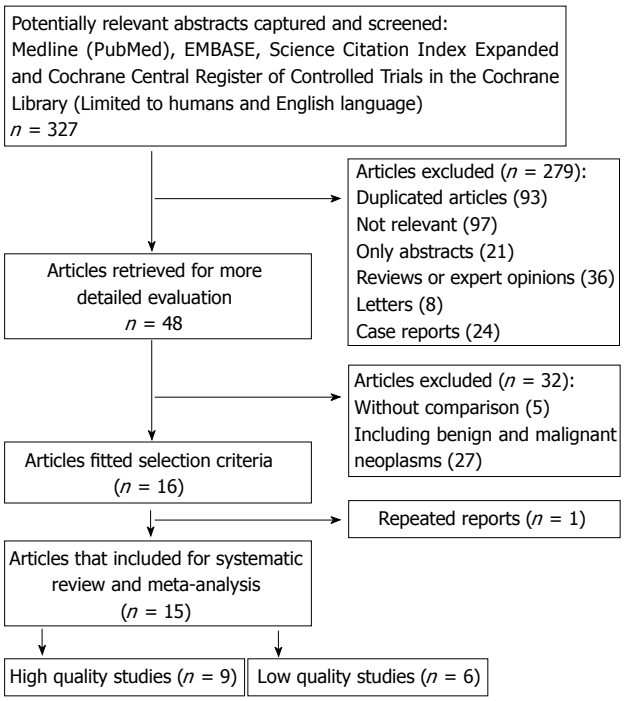


Figure 1 Flow diagram depicting the process of identification and inclusion of selected studies.

base searches. Final inclusion of articles was determined by consensus from two authors; when this failed, a third author adjudicated.

Inclusion and exclusion criteria

Two authors identified and screened the aforementioned databases for potentially eligible studies.

Inclusion criteria: (1) Clear documentation of the operative techniques as "laparoscopic" or "open"; (2) Studies with at least one of the outcomes mentioned; and (3) Where multiple studies came from the same institute and/or authors, either the one of higher quality or the most recent publication was included in the analysis.

Exclusion criteria: (1) Abstracts, letters, editorials, expert opinions, case reports, reviews and studies lacking control groups; (2) Studies with no clearly reported outcomes of interest; (3) Studies dealing with HCC recurrence after hepatectomy; and (4) Studies including patients with benign lesions or other types of malignant liver tumors.

Outcomes of interest

The following outcomes were evaluated in the two approaches.

Operative outcomes: Operative time, intraoperative blood loss and requirement for blood transfusions.

Postoperative outcomes: Hospital stay, liver failure, cirrhotic decompensation/ascites, bile leakage, postoperative bleeding, pulmonary complications (including pleural effusion and pneumonia), intra-abdominal abscess and mortality.

Oncologic outcomes: Positive resection margins and tumor recurrence.

Data extraction and quality assessment

Data were extracted by two independent observers using standardized forms. The recorded data included patient and study characteristics and surgical details. The quality of studies was assessed using the Newcastle-Ottawa Scale[21], by examining three factors: patient selection, comparability of the study groups and assessment of outcome. Studies were matched for age, American Society of Anesthesiologists status, presence of cirrhosis, size of tumor and type of hepatic resection undertaken. The maximum numbers of stars in the selection, comparability, and outcome categories were four, two, and three, respectively. Studies achieving six or more stars were considered to be of higher quality[22]. Only these were included in the final analysis to have the best estimate of the outcome measure.

Statistical analysis

Meta-analysis was performed using Review Manager Version 5.0 software (The Cochrane Collaboration, Oxford, United Kingdom). For continuous variables, treatment effects were expressed as weighted mean difference (WMD) with corresponding 95%CI. For categorical variables, treatment effects were expressed as odds ratio (OR) with corresponding 95%CI. Heterogeneity was evaluated using the χ^2 test, and a P value < 0.1 was considered significant[23]. The fixed-effects model was initially calculated for all outcomes[24]. If the test rejected the assumption of homogeneity of studies, random-effects analysis was performed[25]. Sensitivity analysis were performed by removing individual studies from the data set and analyzing the effect on the overall results to identify sources of significant heterogeneity. Subgroup analysis were also undertaken by including low-quality studies to present cumulative evidence. Funnel plots were constructed to evaluate potential publication bias[26] based on the operative time, hospital stay and tumor recurrence.

RESULTS

Description of included trials in the meta-analysis

The search strategy initially generated 327 relevant clinical trials. Finally, 16 articles[10-12,16-20,27-34] were selected for further investigation. Of these, two studies[12,30] were published by the same institute and had overlapping patient populations; therefore, the higher-quality study[30] was included. In total, 15 non-randomized comparative studies were identified for final inclusion, out of which 9 were found to be of high quality[10,11,17,18,20,28,30,32,33]. These were included in the final analyses. Figure 1 shows the process of selecting comparative studies included in our meta-analysis.

Table 1 Characteristics of studies included in the meta-analysis

Study	Year	Country	Group	*n*	Male/female	Age (yr) (mean ± SD)	Matching[b]	Study quality
Shimada *et al*[27]	2001	Japan	LLR	17	15/2	62 ± 9	1,3,4	*****
			OLR	38	24/14	63 ± 79		
Laurent *et al*[28]	2003	France	LLR	13	10/3	62.6 ± 9.5	1,3,4,5	******
			OLR	14	10/4	65.9 ± 5.5		
Kaneko *et al*[29]	2005	Japan	LLR	30	18/12	59 ± 8	1,2,3,4,5	*****
			OLR	28	18/10	61 ± 10		
Belli *et al*[30]	2007	Italy	LLR	23	13/10	59.5 ± 6.84	1,2,3,4,5	*******
			OLR	23	14/9	62.4 ± 7.7		
Endo *et al*[31]	2009	Japan	LLR	10	8/2	72 ± 4	3,4	****
			OLR	11	8/3	64 ± 2		
Lai *et al*[32]	2009	China	LLR	25	18/7	59 (35-79)[a]	1,3,4	******
			OLR	33	21/12	59 (38-77)[a]		
Sarpel *et al*[11]	2009	United States	LLR	20	15/5	63.8 ± 10.3	1,3,4	*******
			OLR	56	45/11	58.3 ± 11.0		
Aldrighetti *et al*[33]	2010	Italy	LLR	16	11/5	65 ± 10	1,2,3,4,5	********
			OLR	16	12/4	71 ± 6		
Tranchart *et al*[10]	2010	France	LLR	42	15/27	63.7 ± 13.1	1,2,3,4,5	******
			OLR	42	14/28	65.7 ± 7.1		
Nguyen *et al*[34]	2011	United States	LLR	17	12/5	68	1,3,4,5	*****
			OLR	20	12/8	65		
Hu *et al*[16]	2011	China	LLR	30	20/10	46 ± 12	1,3,4	****
			OLR	30	19/11	48 ± 15		
Ker *et al*[19]	2011	China	LLR	116	92/24	58.31 ± 12.7	1,2	****
			OLR	208	156/52	57.9 ± 11.2		
Kim *et al*[20]	2011	South Korea	LLR	26	18/8	57.84 ± 9.66	1,2,3,4,5	******
			OLR	29	20/9	57.08 ± 9.78		
Lee *et al*[17]	2011	China	LLR	33	24/9	59 (36-85)[a]	1,2,4	*******
			OLR	50	40/10	58.5 (32-81)[a]		
Truant *et al*[18]	2011	France	LLR	36	31/5	60.6 ± 10.2	1,2,3,4	*******
			OLR	53	47/6	63.3 ± 7.6		

LLR: Laparoscopic liver resection; OLR: Open liver resection. [a]Median with range; [b]1: Age; 2: American Society of Anesthesiologists physical status score; 3: Presence of cirrhosis; 4: Tumor size; 5: Type of liver resection.

Study and patient characteristics

The characteristics and quality assessments of included studies are shown in Table 1. A total of 550 patients were included: 234 patients in the LLR and 316 patients in the OLR group. The characteristics of patients and surgical details are summarized in Table 2. The sample size of the included studies varied from 21 to 89 patients. The rate of conversion, from laparoscopic to open procedure, ranged from 0% to 19.4%. Patients in most of studies had concurrent hepatitis B infection.

Meta-analysis results

Results of the analyses are shown in Figure 2 and summarized in Table 3.

Operative outcomes: Six high-quality studies[10,11,18,28,30,33] reported mean operation time, analysis of which showed no statistically significant difference between the two groups (patients 354; WMD: 4.69 min; 95%CI: -22.62-32 min; $P = 0.74$). Similarly, six high-quality studies[10,18,20,28,30,33] provided detailed data for estimation of blood loss between the two groups. We found that LLR had significantly less intraoperative blood loss compared to OLR [patients 333; WMD: -129.48 mL; 95%CI: -224.76-(-34.21) mL; $P = 0.008$]. Furthermore, the rate of blood transfusions requirement was identified to be significantly lower in the LLR group as opposed to OLR (trials: 7; patients 416; OR: 0.49; 95%CI: 0.26-0.91; $P = 0.02$). Addition of low-quality trials to these groups did not affect the results.

Postoperative outcomes: Six high-quality studies[10,18,20,28,30,33] reported on length of hospital stay. Pooled outcome measure favored LLR [patients 333; WMD: -3.19 d; 95%CI: -4.09-(-2.28) d; $P < 0.00001$]. A lower incidence of liver failure was observed in patients undergoing LLR (trials 2, patients 116; OR 0.15; 95%CI: 0.02-0.95; $P = 0.04$). The incidence of postoperative ascites in seven high-quality trials (patients 416; OR: 0.32; 95%CI: 0.16-0.61; $P = 0.0006$) was found to be significantly lower in LLR group. Six high-quality trials[10,17,18,20,28,30] revealed no statistically significant difference in the incidence of pulmonary complications between the two groups (patients 384; OR: 0.43; 95%CI: 0.18-1.04; $P = 0.06$). However, when two low-quality trials[27,31] were also pooled together to get a cumulative result, LLR group seemed to have a lower incidence (patients 460; OR: 0.43; 95%CI: 0.19-0.96; $P = 0.04$).

No significant differences were observed between two operative techniques in terms of other postoperative complications, such as bile leakage (trials 3; patients 205; OR: 0.55; 95%CI: 0.10-3.12; $P = 0.50$), postoperative bleeding (trials 5; OR: 0.54; 95%CI: 0.20-1.45; $P = 0.22$) and mortality (trials 5; patients 474; OR: 0.46; 95%CI:

Table 2 Characteristics of patients and surgical details

Study	Group	Cirrhosis *n* (%)	Tumor size (cm)	Type of hepatectomy
Shimada *et al*[27]	LLR	13 (76.4)	2.6 ± 0.9	a = 7, b = 10
	OLR	28 (73.6)	2.5 ± 1.0	NA
Laurent *et al*[28]	LLR	NA	3.35 ± 0.89	a = 3, b = 7, c = 3
	OLR	NA	3.43 ± 1.05	a = 4, b = 7, c = 3
Kaneko *et al*[29]	LLR	13 (43.3)	3.0 ± 0.8	a = 10, b = 20
	OLR	NA	3.1 ± 0.9	a = 8 ,b = 20
Belli *et al*[30]	LLR	23 (100)	3.1 ± 0.7	a = 5, b = 3, c = 15
	OLR	23 (100)	3.24 ± 0.70	a = 6, b = 5, c = 12
Endo *et al*[31]	LLR	6 (60)	3.0 ± 1.5	NA
	OLR	9 (81.8)	4.1 ± 0.8	NA
Lai *et al*[32]	LLR	23 (92)	2.5 (1-7)[1]	a = 6, b = 8, c = 10, d = 1
	OLR	31 (93.9)	2.6 (1-8)[1]	a = 2, b = 18, c = 13
Sarpel *et al*[11]	LLR	9 (45)	4.3 ± 2.1	NA
	OLR	27 (48.2)	4.3 ± 2.2	NA
Aldrighetti *et al*[33]	LLR	9 (56.3)	4 ± 2.2	a = 5, b = 2, c = 9
	OLR	9 (56.3)	4.6 ± 2.5	a = 5, b = 2, c = 9
Tranchart *et al*[10]	LLR	31 (73.8)	3.58 ± 1.75	a = 9, b = 15, c = 10, d = 3, e = 2, f = 3
	OLR	34 (80.9)	3.68 ± 2.09	a = 7, b = 13, c = 10, d = 3, e = 2, f = 7
Nguyen *et al*[34]	LLR	44 (65)	3.0	a = 6, b = 5, e = 6
	OLR	23 (35)	4.5	a = 6, b = 8, e = 6
Hu *et al*[16]	LLR	25 (83.3)	6.7 ± 3.1	NA
	OLR	NA	8.7 ± 2.3	a = 10, b = 20
Ker *et al*[19]	LLR	NA	2.5 ± 1.2	a = 7, c = 97, e = 4, g = 8
	OLR	NA	5.4 ± 3.5	NA
Kim *et al*[20]	LLR	NA	3.15 (1-8)[1]	a = 4, b = 4, c = 13, d = 4, e = 1
	OLR	NA	3.6 (1-19)[1]	a = 3, b = 10, c = 9, d = 5, e = 2
Lee *et al*[17]	LLR	28 (84.8)	2.5 (1.5-9)[1]	a = 18, h = 15
	OLR	32 (64)	2.9 (1.2-9)[1]	a = 10, h = 40
Truant *et al*[18]	LLR	NA	2.9 ± 1.2	a = 22, b or f = 14
	OLR	NA	3.1 ± 1.2	a = 26, b or f = 27

[1]Median and (range). LLR: Laparoscopic liver resection; OLR: Open liver resection; NA: Not available; a: Left lateral segmentectomy; b: Segmentectomy; c: Subsegmentectomy; d: Right hepatectomy; e: Left hepatectomy; f: Bisegmentectomy; g: Right anterior sectorectomy; h: Nonanatomical resection.

0.14-1.51; $P = 0.20$).

Two high-quality trials[20,30] reported intra-abdominal abscess formation in their patient populations. However, one of these did not have any events in both the groups and was subsequently excluded. A subgroup analysis was therefore undertaken including a low-quality study, which also did not show an association of intra-abdominal abscess formation with the type of operative technique (patients 122; OR: 0.72; 95%CI: 0.12-4.54; $P = 0.73$).

Oncologic outcomes: We did not find any significant differences in the rate of positive margins (trials 4; patients 287; OR: 0.59; 95%CI: 0.21-1.62; $P = 0.31$) and tumor recurrence (trials 6; patients 416; OR: 0.95; 95%CI: 0.62-1.46; $P = 0.81$).

Sensitivity and subgroup analysis

Sensitivity analyses were carried out by excluding each individual study from each outcome measure. These exclusions did not alter the results obtained from cumulative analyses. Additionally, the pooled result of included outcomes was not affected, when either fixed effects or random effects models were used. Subgroup analyses were undertaken for all outcome measures by including low-quality studies as well. These are summarized in Table 3.

Publication bias

The funnel plot was based on the operation time, hospital stay and tumor recurrence, which is shown in Figure 3. As no study lies outside the limits of the 95%CI, there was no evidence of publication bias.

DISCUSSION

LLR is a challenging technique for surgeons as the liver has unique anatomical features which present technical difficulties for parenchymal transections-massive hemorrhage and bile leak from intrahepatic vessels[11,29]. Presence of cirrhosis in patients undergoing LLR makes parenchymal transaction an even more delicate and demanding procedure[11]. Rare but fatal complications such as a gas embolism caused by the pneumoperitoneum through hepatic venous branches on the hepatic stump during parenchymal division of the liver have also been reported[27]. On the contrary, increased experience, technical refinement and improvement in surgical equipment have increased the safety of liver resection as a curative treatment for benign or malignant liver lesions[35-38]. In spite of these advancements, LLR has not been very popular for HCC, partly because of the controversies related to resection margins, tumor seeding, incision related metastasis, and long-term survival[32]. However, the difference in outcomes between LLR and OLR in HCC has not been evaluated in a randomized controlled trial. Most reported studies are retrospective, single-institution series with a small number of patients, which makes it difficult to interpret outcomes appropriately. In order to overcome these limitations, we have endeavored to pool all the relevant available data and perform a meta-analysis. Although our result is similar to previously reports[13-15] in some aspects, such as operative blood loss, blood transfusion requirement, length of hospital stay and tumor recurrence, our analysis included more high-quality case-matched studies as well as more patients and therefore, provides an up to date and high-quality evidence regarding the perioperative and long-term outcomes of patients with HCC undergoing LLR *vs* OLR.

We found no significant difference in the 30-d mortality between the two groups. The results from this analysis indicate that LLR was successfully completed in most patients, with a rate of conversion to open surgery ranging from 0% to 19.4%. These results point towards the feasibility of LLR for patients with HCC.

There was no significant difference in operative time between the two techniques, based on our analysis, which can be explained by current advances in surgical instru-

Table 3 Results of meta-analysis comparing laparoscopic *vs* open hepatectomy (only high-quality studies)

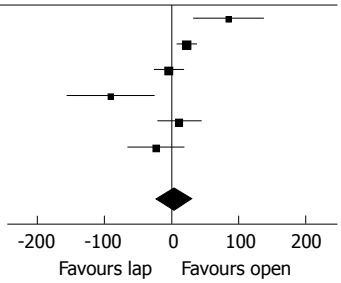
Outcome of interest	No. of studies	No. of patients	OR/WMD	95%CI	*P* value	Heterogeneity *P* value	I^2 (%)
Operative outcomes							
Operation time (min)	6	354	4.69	-22.62, 32.00	0.74	0.0002	79
Intraoperative blood loss (mL)	6	333	-129.48	-224.76, -34.21	0.008	0.01	67
Blood transfusions requirement	7	416	0.49	0.26, 0.91	0.02	0.89	0
Postoperative outcomes							
Liver failure	2	116	0.15	0.02, 0.95	0.04	1.00	0
Cirrhotic decompensation/ascites	7	416	0.32	0.16, 0.61	0.001	0.95	0
Bile leakage	3	205	0.55	0.10, 3.12	0.50	0.86	0
Postoperative bleeding	5	287	0.54	0.20, 1.45	0.22	0.83	0
Pulmonary complications	6	384	0.43	0.18, 1.04	0.06	0.46	0
Intra-abdominal abscess	2	101	0.21	0.01, 4.53	0.32	-	-
Mortality	8	474	0.46	0.14, 1.51	0.20	0.64	0
Hospital stay	6	333	-3.19	-4.09, -2.28	< 0.00001	0.91	0
Oncologic outcomes							
Surgery margin positive rate	5	287	0.59	0.21, 1.62	0.31	0.65	0
Tumor recurrence	7	416	0.95	0.62, 1.46	0.81	0.93	0

WMD: Weighted mean difference; OR: Odds ratio.

A Operative time

Study or subgroup	Lap mean	Lap SD	Lap Total	Open mean	Open SD	Open Total	Weight	Mean difference IV, radom, 95%CI
Laurent 2003	267	79	13	182	57	14	12.8%	85.00 (32.70, 137.30)
Belli 2007	148	29.73	23	125.21	17.48	23	22.6%	22.79 (8.70, 36.88)
Sarpei 2009	161	37	20	165	53	56	20.9%	-4.00 (-25.35, 17.35)
Aldrighetti 2010	150	57	16	240	121	16	10.1%	-90.00 (-155.54, -24.46)
Tranchart 2010	233.1	92.7	42	221.8	46.3	42	18.2%	11.30 (-20.04, 42.64)
Truant 2011	193.4	104	36	215.8	88.7	53	15.4%	-22.40 (-63.93, 19.13)
Total (95%CI)			150			204	100.0%	4.69 (-22.62, 32.00)

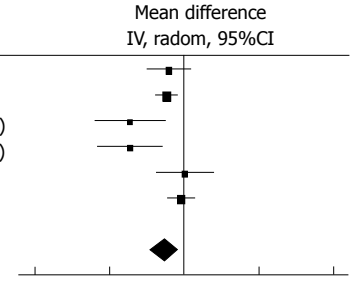
Heterogeneity: τ^2 = 809.09; χ^2 = 23.83; df = 5 (P = 0.0002); I^2 = 79%
Test for overall effect: Z = 0.34 (P = 0.74)



Intraoperative blood loss

Study or subgroup	Lap mean	Lap SD	Lap Total	Open mean	Open SD	Open Total	Weight	Mean difference IV, radom, 95%CI
Laurent 2003	620	130	13	720	240	14	17.1%	-100.00 (-244.22, 44.22)
Belli 2007	260	127	23	376.95	114.32	23	24.5%	-116.95 (-186.78, -47.12)
Aldrighetti 2010	258	186	16	617	433	16	10.6%	-359.00 (-589.91, -128.09)
Tranchart 2010	364.3	435.7	42	723.7	559.5	42	11.6%	-359.40 (-573.86, -144.94)
Truant 2011	452.2	442	36	447.2	449.8	53	13.4%	5.00 (-183.44, 193.44)
Kim 2011	70	150	26	90	182	29	22.7%	-20.00 (-107.82, 67.82)
Total (95%CI)			156			177	100.0%	-129.48 (-224.76, -34.21)

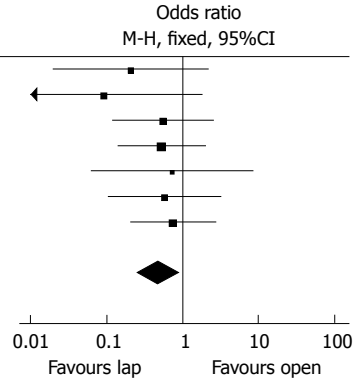
Heterogeneity: τ^2 = 8380.19; χ^2 = 15.06; df = 5 (P = 0.01); I^2 = 67%
Test for overall effect: Z = 2.66 (P = 0.008)



Blood transfusions requirement

Study or subgroup	Lap Events	Lap Total	Open Events	Open Total	Weight	Odds ratio M-H, fixed, 95%CI
Laurent 2003	1	13	4	14	12.1%	0.21 (0.02, 2.18)
Belli 2007	0	23	4	23	15.0%	0.09 (0.00, 1.82)
Aldrighetti 2010	4	16	6	16	15.3%	0.56 (0.12, 2.54)
Tranchart 2010	4	42	7	42	21.5%	0.53 (0.14, 1.95)
Truant 2011	1	36	2	53	5.3%	0.73 (0.06, 8.35)
Lee 2011	2	33	5	50	12.7%	0.58 (0.11, 3.19)
Kim 2011	5	26	7	29	18.2%	0.75 (0.21, 2.73)
Total (95%CI)		189		227	100.0%	0.49 (0.26, 0.91)
Total events	17		35			

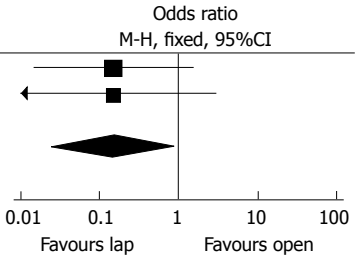
Heterogeneity: χ^2 = 2.31; df = 6 (P = 0.89); I^2 = 0%
Test for overall effect: Z = 2.26 (P = 0.02)



B Liver failure

Study or subgroup	Lap Events	Lap Total	Open Events	Open Total	Weight	Odds ratio M-H, fixed, 95%CI
Laurent 2003	1	13	5	14	55.2%	0.15 (0.01, 1.52)
Truant 2011	0	36	4	53	44.8%	0.15 (0.01, 2.89)
Total (95%CI)		49		67	100.0%	0.15 (0.02, 0.95)
Total events	1		9			

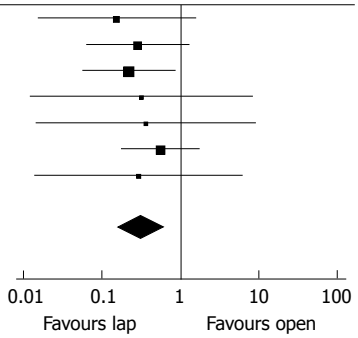
Heterogeneity: χ^2 = 0.00; df = 1 (P = 1.00); I^2 = 0%
Test for overall effect: Z = 2.02 (P = 0.04)



Postoperative ascites

Study or subgroup	Lap Events	Lap Total	Open Events	Open Total	Weight	Odds ratio M-H, fixed, 95%CI
Laurent 2003	1	13	5	14	12.8%	0.15 (0.01, 1.52)
Belli 2007	3	23	8	23	20.0%	0.28 (0.06, 1.24)
Tranchart 2010	3	42	11	42	29.4%	0.22 (0.06, 0.85)
Aldrighetti 2010	0	16	1	16	4.2%	0.31 (0.01, 8.28)
Kim 2011	0	26	1	29	4.0%	0.36 (0.01, 9.19)
Truant 2011	5	36	12	53	24.0%	0.55 (0.18, 1.73)
Lee 2011	0	33	2	50	5.7%	0.29 (0.01, 6.23)
Total (95%CI)		189		227	100.0%	0.32 (0.16, 0.61)
Total events	12		40			

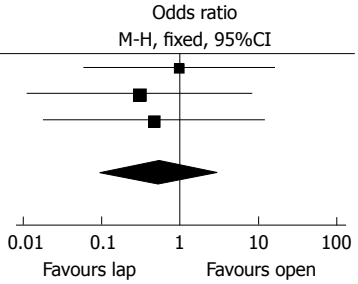
Heterogeneity: χ^2 = 1.64; df = 6 (P = 0.95); I^2 = 0%
Test for overall effect: Z = 3.42 (P = 0.0006)



Bile leakage

Study or subgroup	Lap Events	Lap Total	Open Events	Open Total	Weight	Odds ratio M-H, fixed, 95%CI
Tranchart 2010	1	42	1	42	26.9%	1.00 (0.06, 16.53)
Aldrighetti 2010	0	16	1	16	40.0%	0.31 (0.01, 8.28)
Truant 2011	0	36	1	53	33.1%	0.48 (0.02, 12.10)
Total (95%CI)		94		111	100.0%	0.55 (0.10, 3.12)
Total events	1		3			

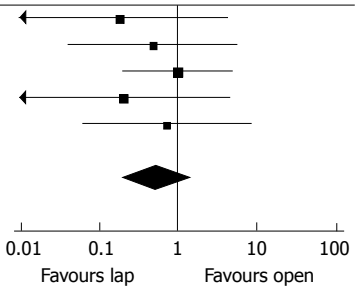
Heterogeneity: χ^2 = 0.29; df = 2 (P = 0.86); I^2 = 0%
Test for overall effect: Z = 0.67 (P = 0.50)



Postoperative bleeding

Study or subgroup	Lap Events	Lap Total	Open Events	Open Total	Weight	Odds ratio M-H, fixed, 95%CI
Laurent 2003	0	13	2	14	20.8%	0.19 (0.01, 4.25)
Tranchart 2010	1	42	2	42	17.5%	0.49 (0.04, 5.59)
Aldrighetti 2010	4	16	4	16	26.8%	1.00 (0.20, 4.95)
Kim 2011	0	26	2	29	20.8%	0.21 (0.01, 4.53)
Truant 2011	1	36	2	53	14.1%	0.73 (0.06, 8.35)
Total (95%CI)		133		154	100.0%	0.54 (0.20, 1.45)
Total events	6		12			

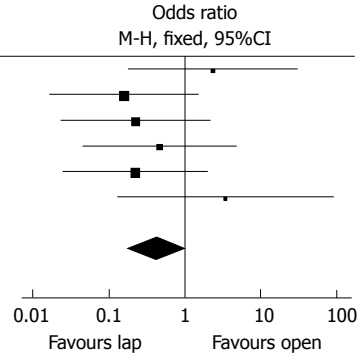
Heterogeneity: χ^2 = 1.45; df = 4 (P = 0.83); I^2 = 0%
Test for overall effect: Z = 1.22 (P = 0.22)



Pulmonary complications

Study or subgroup	Lap Events	Lap Total	Open Events	Open Total	Weight	Odds ratio M-H, fixed, 95%CI
Laurent 2003	2	13	1	14	4.8%	2.36 (0.19, 29.71)
Belli 2007	1	23	5	23	28.2%	0.16 (0.02, 1.53)
Tranchart 2010	1	42	4	42	23.1%	0.23 (0.02, 2.17)
Truant 2011	1	36	3	53	13.9%	0.48 (0.05, 4.77)
Lee 2011	1	33	6	50	27.3%	0.23 (0.03, 2.00)
Kim 2011	1	26	0	29	2.6%	3.47 (0.14, 88.99)
Total (95%CI)		173		211	100.0%	0.43 (0.18, 1.04)
Total events	7		19			

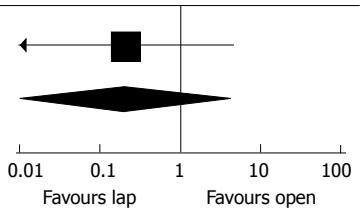
Heterogeneity: χ^2 = 4.67; df = 5 (P = 0.46); I^2 = 0%
Test for overall effect: Z = 1.88 (P = 0.06)



Intraabdominal abscess

Study or subgroup	Lap Events	Lap Total	Open Events	Open Total	Weight	Odds ratio M-H, fixed, 95%CI
Belli 2007	0	23	0	23		Not estimable
Kim 2011	0	26	2	29	100.0%	0.21 (0.01, 4.53)
Total (95%CI)		49		52	100.0%	0.21 (0.01, 4.53)
Total events	0		2			

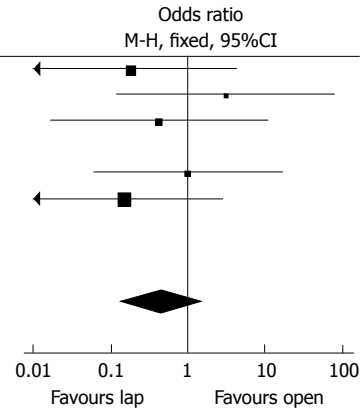
Heterogeneity: Not applicable
Test for overall effect: $Z = 1.00$ ($P = 0.32$)



Mortality

Study or subgroup	Lap Events	Lap Total	Open Events	Open Total	Weight	Odds ratio M-H, fixed, 95%CI
Laurent 2003	0	13	2	14	26.9%	0.19 (0.01, 4.25)
Belli 2007	1	23	0	23	5.4%	3.13 (0.12, 81.00)
Lai 2009	0	25	1	33	14.7%	0.42 (0.02, 10.87)
Aldrighetti 2010	0	16	0	16		Not estimable
Tranchart 2010	1	42	1	42	11.3%	1.00 (0.06, 16.53)
Truant 2011	0	36	4	53	41.7%	0.15 (0.01, 2.89)
Kim 2011	0	26	0	29		Not estimable
Lee 2011	0	33	0	50		Not estimable
Total (95%CI)		214		260	100.0%	0.46 (0.14, 1.51)
Total events	2		8			

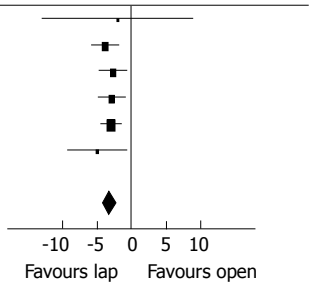
Heterogeneity: $\chi^2 = 2.51$; df = 4 ($P = 0.64$); $I^2 = 0\%$
Test for overall effect: $Z = 1.28$ ($P = 0.20$)



Hospital stay

Study or subgroup	Lap mean	Lap SD	Lap Total	Open mean	Open SD	Open Total	Weight	Odds ratio IV, fixed, 95%CI
Laurent 2003	15.3	8.6	13	17.3	18.9	14	0.7%	-2.00 (-12.95, 8.95)
Belli 2007	8.2	2.6	23	12.04	3.93	23	22.2%	-3.84 (-5.77, -1.91)
Aldrighetti 2010	6.3	1.7	16	9	3.8	16	19.8%	-2.70 (-4.74, -0.66)
Tranchart 2010	6.7	5.9	42	9.6	3.4	42	19.4%	-2.90 (-4.96, -0.84)
Truant 2011	6.5	2.7	36	9.5	4.8	53	33.6%	-3.00 (-4.56, -1.44)
Kim 2011	11.08	4.96	26	16.07	10.697	29	4.4%	-4.99 (-9.32, -0.66)
Total (95%CI)			156			177	100.0%	-3.19 (-4.09, -0.66)

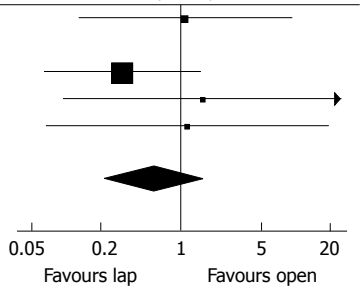
Heterogeneity: $\chi^2 = 1.50$; df = 5 ($P = 0.91$); $I^2 = 0\%$
Test for overall effect: $Z = 6.89$ ($P < 0.00001$)



C Positive resection margins

Study or subgroup	Lap Events	Lap Total	Open Events	Open Total	Weight	Odds ratio M-H, fixed, 95%CI
Laurent 2003	2	13	2	14	15.6%	1.09 (0.13, 9.12)
Belli 2007	0	23	0	23		Not estimable
Sarpel 2009	2	20	15	56	68.2%	0.30 (0.06, 1.47)
Lee 2011	1	33	1	50	7.4%	1.53 (0.09, 25.37)
Kim 2011	1	26	1	29	8.7%	1.12 (0.07, 18.86)
Total (95%CI)		115		172	100.0%	0.59 (0.21, 1.62)
Total events	6		19			

Heterogeneity: $\chi^2 = 1.65$; df = 3 ($P = 0.65$); $I^2 = 0\%$
Test for overall effect: $Z = 1.03$ ($P = 0.31$)



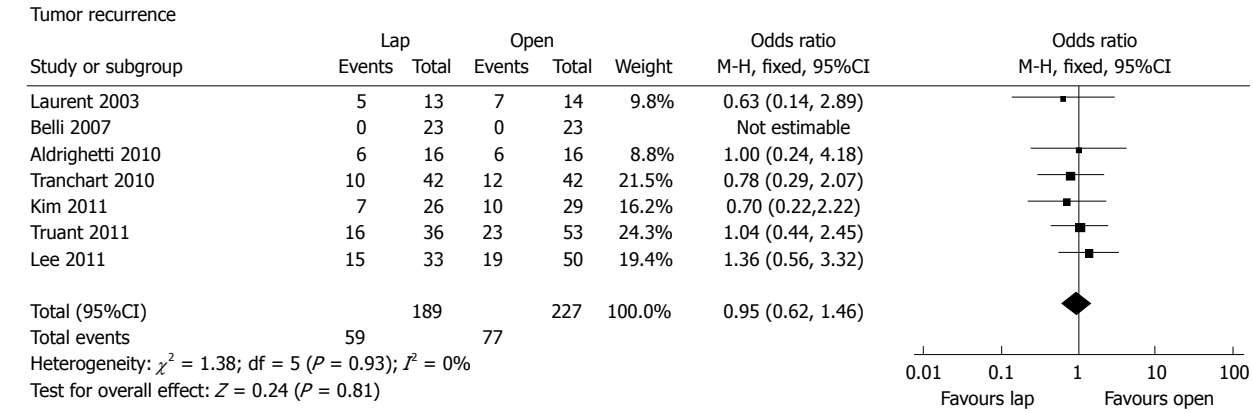


Figure 2 Forest plots demonstrating operative, postoperative and oncologic outcomes. A: Forest plots illustrating results of operative outcomes in the form of meta-analysis comparing laparoscopic *vs* open resection for hepatocellular carcinoma (high-quality studies only); B: Forest plots illustrating results of postoperative outcomes in the form of meta-analysis comparing laparoscopic *vs* open resection for hepatocellular carcinoma (high-quality studies only); C: Forest plots illustrating results of oncologic outcomes in the form of meta-analysis comparing laparoscopic *vs* open resection for hepatocellular carcinoma (high quality studies only). Pooled weighted mean difference or odds ratio with 95%CI was calculated using the fixed-effects or random effects model. IV: Iverse variance; M-H: Mantel-Haenszel.

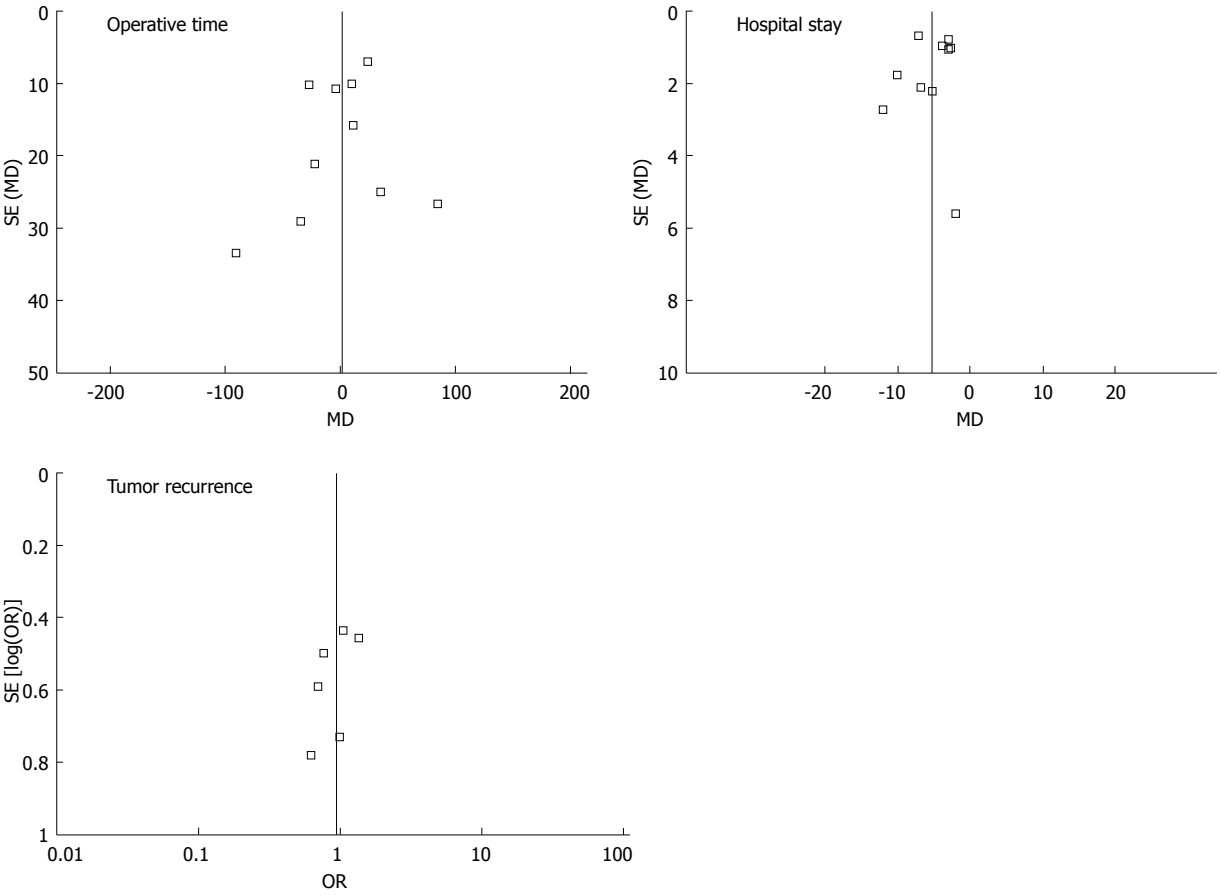


Figure 3 Funnel plot to investigate publication bias. The laparoscopic *vs* the open group: A funnel plot showing the operation time, hospital stay and tumor recurrence. OR: Odds ratio; MD: Mean difference.

mentation and technology, as well as surgeons' experience and learning curve[39]. Our results demonstrate that LLR is associated with significantly less intraoperative blood loss and blood transfusion requirement, which can partly be explained by the hemostatic effect of pneumoperitoneum on the hepatic vein branches[30,40] and also image magnification during LLR[33]. There have been some reports in literature indicating that significant intraoperative blood loss and blood transfusion are associated with recurrence and survival rates after resection of HCC[41-43]. Hence reduced blood loss in LLR is favorable. Results from this meta-analysis also reveal a significant reduction in the postoperative hospital stay in the LLR group. These findings are consistent with laparoscopic procedures

where patients have faster ambulation, early oral intake and reduced analgesic requirements[11,33].

There is growing evidence to suggest that LLR is associated with less postoperative morbidity particularly with regards to developing postoperative ascites and liver failure. The reduction in the incidence of postoperative ascites in LLR might be due to preservation of the abdominal wall collateral circulation, by avoiding long abdominal incisions and preservation of the round ligament, which may contain significant collateral veins, thereby reducing portal hypertension and intraoperative fluid requirements[44]. Other favorable factors associated with LLR include less frequent mobilization and manipulation of the liver, reduced fluid requirements, decreased blood loss, early ambulation and oral food intake and reduced third space accumulation leading to hyperaldosteronism[28,30,45-47].

Incomplete tumor resection with positive resection margins is perceived to be a potential disadvantage in LLR[33]. However, our results reveal no significant difference in the margin positive rate between the LLR and OLR groups. Further analysis revealed no difference in recurrence between the two groups. These findings can be attributed to the use of intraoperative ultrasonography in LLR or OLR. Intraoperative ultrasonography is a sensitive tool for accurate identification of lesions and orientation of borders for non-tumorous tissue[48,49]. The other consideration for laparoscopic resection of malignancies is the potential of peritoneal dissemination, or port-site metastasis[50-52]. However we did not encounter any case of peritoneal dissemination or port-site metastasis in our analysis.

Although our analysis shows apparent advantages of LLR over OLR for HCC, it is important to highlight that most of the patients included in our meta-analysis underwent segmentectomy or subsegmentectomy for peripheral lesions located in the anterolateral segments of the liver. Although it is encouraging that our results have been consistent throughout the sensitivity analyses, this meta-analysis also has some limitations which should be considered when interpreting its results and warrants a discussion. Firstly, all of the studies included were non-randomized, retrospective trials, which inevitably add a degree of selection bias to the results and can lead to over/under estimation of the measured effect. Since factors such as tumor location, extent of liver cirrhosis and tumor size are important determinants of outcome, we matched the two groups based on these important factors to eliminate bias and improve the validity of our results[53].

Secondly, we observed some heterogeneity in certain outcome measures. This might be explained by differences in surgical techniques, retrospective nature of the studies, and limited blinded outcome assessment in some of the trials. However investigation of heterogeneity using meta-regression was not possible due to small number of studies.

Thirdly, there was inconsistency in the definition of some outcomes in different studies, making it difficult to pool the results together. Using standardized guidelines to report outcomes can potentially overcome this problem and would allow more studies to be included in meta-analyses, leading to more reliable conclusions.

Finally, it is important to note that surgeons' experience and volume of cases operated in a particular hospital may affect these outcome measures tremendously. Unfortunately, none of the studies included in this analysis provided details of these factors and therefore, we were unable to assess the effect in such settings. Future trials should carefully consider such stratification while designing their studies and interpreting their data.

In conclusion, the results of this comprehensive, high-quality up to date meta-analysis indicate that LLR is feasible and safe for the treatment of HCC. LLR should be performed in selected patients by expert surgeons in high volume centers. Further research by undertaking well designed, prospective randomized controlled trials can confirm the advantages of LLR for the management of HCC.

COMMENTS

Background

Laparoscopic liver resection (LLR) is an attractive treatment for liver benign tumor comparing with open liver resection (OLR) because of good cosmetic results and less trauma, but its role remains controversial when LLR is applied to hepatocellular carcinoma (HCC) because of a lack of high-quality randomized controlled trials in this area.

Research frontiers

In order to compare the safety and effectiveness between the LLR and OLR, the meta-analysis was used to evaluate operative, postoperative and oncologic outcomes of these two surgical methods for HCC in this study.

Innovations and breakthroughs

Although previous meta-analysis had compared the outcomes of these two surgical methods, which included a limited number of studies with fewer cases, many high-quality studies with more participants have been published since. Therefore, it is important to provide an up to date analysis of these outcomes. This meta-analysis reported that LLR had significant advantage over OLR in terms of intraoperative blood loss, blood transfusions requirement, hospital stay, postoperative ascites and liver failure compared with OLR for HCC. Meanwhile, incidences of operation time, bile leakage, postoperative bleeding, pulmonary complications, intra-abdominal abscess, mortality, positive resection margins and tumor recurrence were similar between LLR and OLR.

Applications

The results of this meta-analysis show that LLR appears to be a safe and feasible option for HCC in selected patients based on current evidence. Therefore, LLR may be an alternative treatment for HCC. However, the experience of the operating surgeon and volume of operated cases in a particular centre has to be taken into consideration.

Terminology

HCC is the fifth most common primary cancer worldwide with high malignant potential.

Peer review

The paper investigates the safety and effectiveness of LLR on HCC. The statistical analysis used in the study is appropriate and the results suggest that there are some advantages in LLR. This paper should be of interest to surgeons in the field of the hepato-biliary-pancreatic surgery worldwide.

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S- Editor Gou SX **L- Editor** Ma JY **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v18.i45.6669

World J Gastroenterol 2012 December 7; 18(45): 6669-6673
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Small serotonin-positive pancreatic endocrine tumors caused obstruction of the main pancreatic duct

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Received: May 22, 2012 Revised: August 1, 2012
Accepted: August 3, 2012
Published online: December 7, 2012

Abstract

We report 2 cases of pancreatic endocrine tumors that caused obstruction of the main pancreatic duct (MPD). A 49-year-old asymptomatic man was referred to our institution because dilation of the MPD was revealed by abdominal ultrasonography (US). No tumor was detected by endoscopic ultrasonography, computed tomography (CT), and magnetic resonance imaging (MRI). The diameter of the MPD was > 20 mm at the body, and no dilation was noted at the head. Although malignancy was not confirmed through cytology or imaging, pancreatic cancer was strongly suspected. Pancreaticoduo- denectomy was performed. Pathological and immunohistochemical examination revealed a 5 mm × 3 mm serotonin-positive endocrine tumor. Fibrosis was present around the MPD and seemed to cause stricture. A 32-year-old asymptomatic man had elevated serum amylase, and US demonstrated dilation of the MPD. No tumor was detected by CT and MRI. Pancreatic cancer was suspected due to stricture and dilation of the MPD. Pancreatectomy of middle part of pancreas was performed. Pathological and immunohistochemical examination revealed a serotonin-positive endocrine tumor sized 5 mm × 4 mm. We report 2 cases of serotonin-positive pancreatic endocrine tumors that caused stricture of the MPD in spite of the small size of the tumor.

Key words: Serotonin; Pancreatic endocrine tumor; Main pancreatic duct; Obstruction; Dilatation

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Ogawa M, Kawaguchi Y, Maruno A, Ito H, Nakagohri T, Hirabayashi K, Yamamuro H, Yamashita T, Mine T. Small serotonin-positive pancreatic endocrine tumors caused obstruction of the main pancreatic duct. *World J Gastroenterol* 2012; 18(45): 6669-6673 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i45/6669.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i45.6669

INTRODUCTION

Pancreatic endocrine tumors, also known as islet cell tumors, are rare neoplasms and occur in approximately

1 of 100 000 people, representing 1% to 10% of all pancreatic neoplasms[1-3]. Some pancreatic endocrine tumors release hormones into the blood stream that cause clinical syndromes, whereas others are non-syndromic and present as mass lesion[4,5]. The overall prevalence of functional pancreatic endocrine tumors is reported to be approximately 10/1 000 000. In contrast, the prevalence of pancreatic endocrine tumors reported by autopsy studies is higher (0.5% to 1.5%)[6]. Multi-detector row computed tomography (CT) plays an important role in the diagnosis and staging of both syndromic and non-syndromic pancreatic endocrine tumors. In general, syndromic pancreatic endocrine tumors are less than 3 cm in size. They are typically strongly enhanced and usually best seen on CT scans obtained during the arterial phase. Compared to syndromic pancreatic endocrine tumors, non-syndromic pancreatic endocrine tumors tend to be larger at presentation and are more likely to be cystic or necrotic[7]. In previous studies, endoscopic retrograde pancreatography (ERP) has been used to detect main pancreatic duct (MPD) stenosis and dilation of the upstream pancreatic duct caused by pancreatic endocrine tumors[8,9]. In addition, pancreatic endocrine tumors with serotonin production is suggested to be associated with prominent stromal fibrosis, which can extend to the MPD, causing ductal stenosis and upstream dilatation of the duct and/or upstream pancreatic atrophy[10,11]. We recently encountered 2 cases of small pancreatic endocrine tumors that revealed serotonin-positive by immnohistochemical staining causing obstruction of the MPD.

CASE REPORT

Case 1

A 49-year-old asymptomatic man was referred to our institution because dilatation of the MPD was revealed by abdominal ultrasonography (US). However, no tumor was observed by endoscopic ultrasonography (EUS), CT, and magnetic resonance imaging (MRI). The diameter of the MPD was > 20 mm at the body and no dilation was noted at the head. Endoscopic retrograde cholangiopancreatography (ERCP) was performed; the MPD showed crab-like appearance (Figure 1). Cytology was benign. Although we could not confirm malignancy through cytology or imaging, pancreatic cancer was strongly suspected. Pancreaticoduodenectomy was performed, and pathological examination revealed a 5 mm × 3 mm tumor. Fibrosis was present around the MPD and seemed to cause stricture (Figure 2).

Immunohistochemical staining of tumor samples was positive for chromogranin A, synaptophysin, and serotonin (Figure 3). The MIB-1 (Ki-67) labeling index was less than 1%. We made the diagnosis of neuroendocrine tumor of grade 1 (NET G1).

Case 2

A 32-year-old man with no symptom had elevated serum amylase, and US demonstrated dilation of the MPD. No tumor but MPD stenosis and dilatation was revealed by MRI. EUS demonstrated presence of a small pancreatic tumor. ERCP revealed MPD stenosis and upstream dilatation (Figure 4). Cytology was benign.

Pancreatectomy of middle part of pancreas was performed and pathological examination revealed an endocrine tumor sized 5 mm × 4 mm. Tumors with stromal fibrosis can cause stenosis of the pancreatic duct. Immunohistochemical staining of the tumor cells was positive for chromogranin A, synaptophysin, and serotonin (Figure 5). The Ki-67 labeling index was less than 1%, and we made the diagnosis of NET G1.

DISCUSSION

Pancreatic endocrine neoplasms that produce serotonin, including carcinoid tumors, account for only a small portion of pancreatic endocrine neoplasms. Compared with other well-differentiated endocrine neoplasms of the pancreas, pancreatic carcinoid tumors are associated with a higher rate of malignant behavior[12]. These pancreatic neoplasms may have the poor prognosis since they are usually found at an advanced stage, after distant metastases have occurred, and the patient has developed carcinoid syndrome. The problem is that the mass lesion is often asymptomatic and indistinct at early stages. In addition, Shi *et al*[11] reported that in serotonin-producing tumors, the neoplasm was subtle or unapparent on CT images; only marked dilatation of the upstream pancreatic duct or marked atrophy of the upstream pancreas was visible. Isolated reports of an association of pancreatic carcinoid tumor with dilatation of the pancreatic duct have been described before. Nagai *et al*[10] reported a case of pancreatic carcinoid tumor with obstructive pancreatitis. ERP revealed MPD stenosis and dilatation of the upstream pancreatic duct. They suggested that the pancreatic carcinoid tumor obstructing the pancreatic duct might have arisen from argentaffin cells located in the MPD. However, no clear relationship between pancreatic endocrine neoplasms and pancreatic duct stenosis has been described. Takaji *et al*[13] reported that 3 of 4 cases showed MPD dilatation upstream of the tumor. Our 2 cases were similar to these reports; the tumors were subtle on CT or MRI images and a markedly dilated MPD was noted, and they had no symptoms as carcinoid syndrome. We decided to perform the pancreatic resection because there was a possibility of pancreatic duct dilation was caused by a tumor. As a result, we could treat the pancreatic endocrine tumor in early stage. We observed that the serotonin immunoreactivity correlated with the degree of stromal fibrosis and that stromal fibrosis caused MPD stenosis. Carcinoid tumors of the midgut, in which serotonin is the predominant hormone secreted by neoplastic cells, are usually associated with extensive fibrosis[14]. In addition, serotonin has been shown to stimulate fibroblast mitosis in cell cultures[15]. Recently, serotonin has been shown to play a crucial role in the progression of liver fibrosis by enhancing

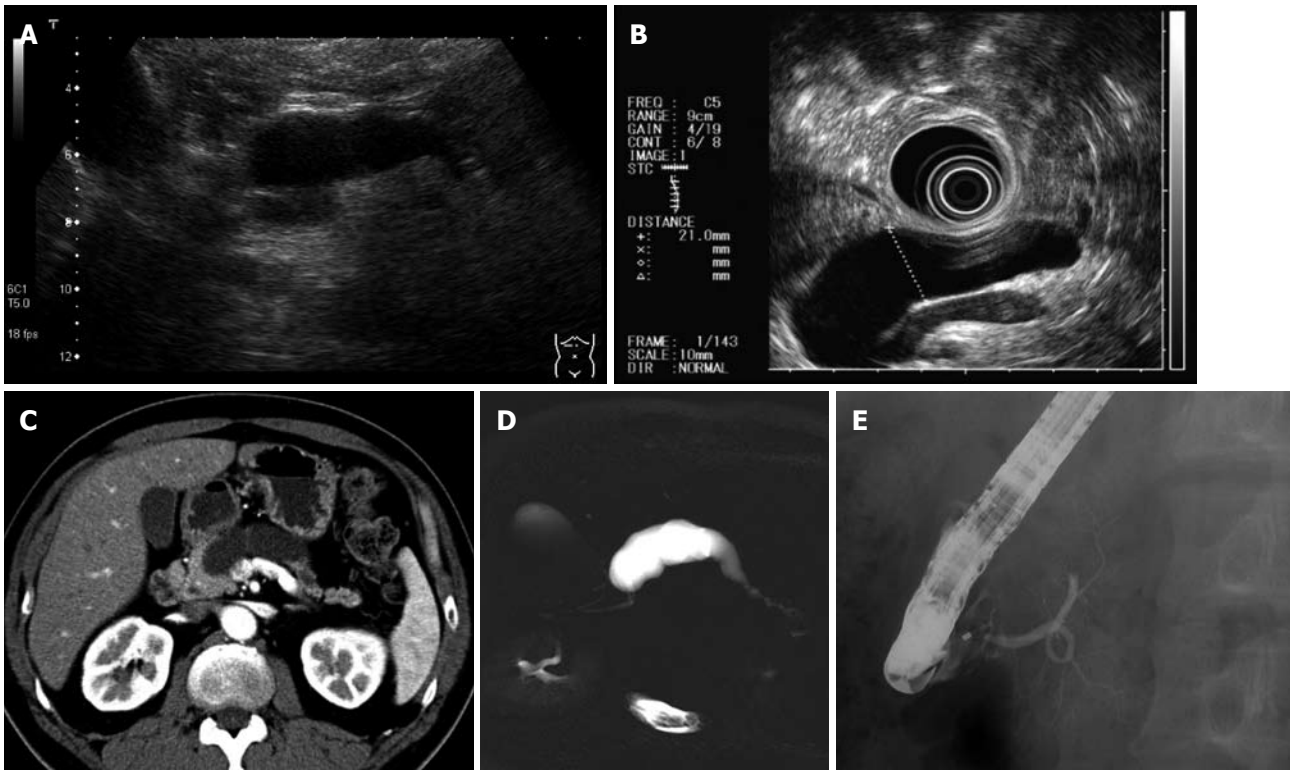

Figure 1 No tumor was detected by endoscopic ultrasonography, computed tomography and magnetic resonance imaging. The diameter of the main pancreatic duct was > 20 mm at the body. A: Ultrasonography; B: Endoscopic ultrasonography; C: Computed tomography; D: Magnetic resonance cholangiopancreatography; E: Endoscopic retrograde pancreatography.

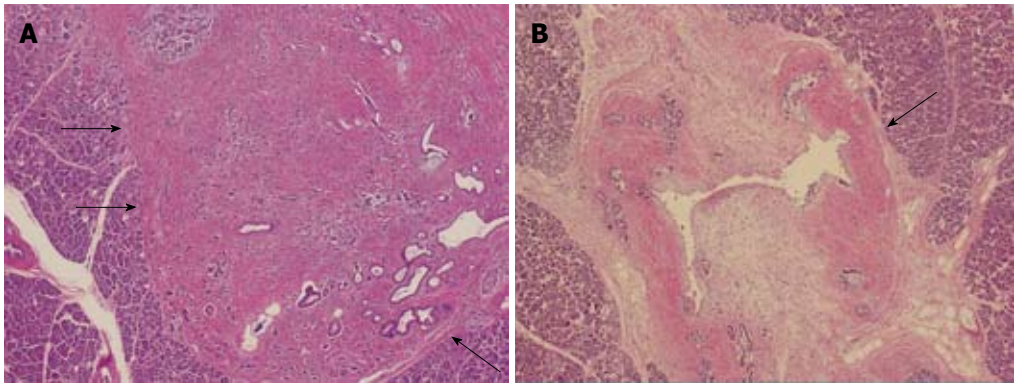

Figure 2 Pathological findings (hematoxylin/eosin staining). A: A 5 mm × 3 mm tumor was detected (arrows); B: Fibrosis was present around the main pancreatic duct (arrow), and it seemed to cause stricture.

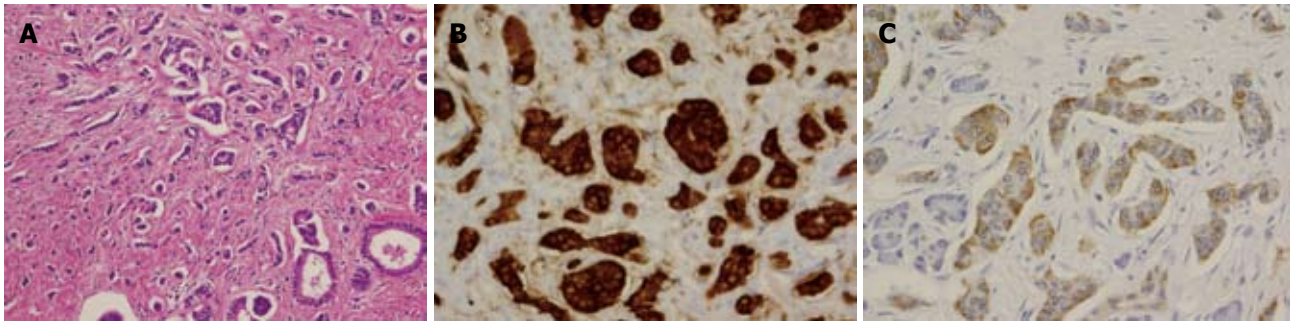

Figure 3 Immunohistochemical staining was positive for chromogranin A, synaptophysin and serotonin. A: Hematoxylin/eosin staining; B: Chromogranin A; C: Serotonin. Ki-67 labeling index was less than 1%.

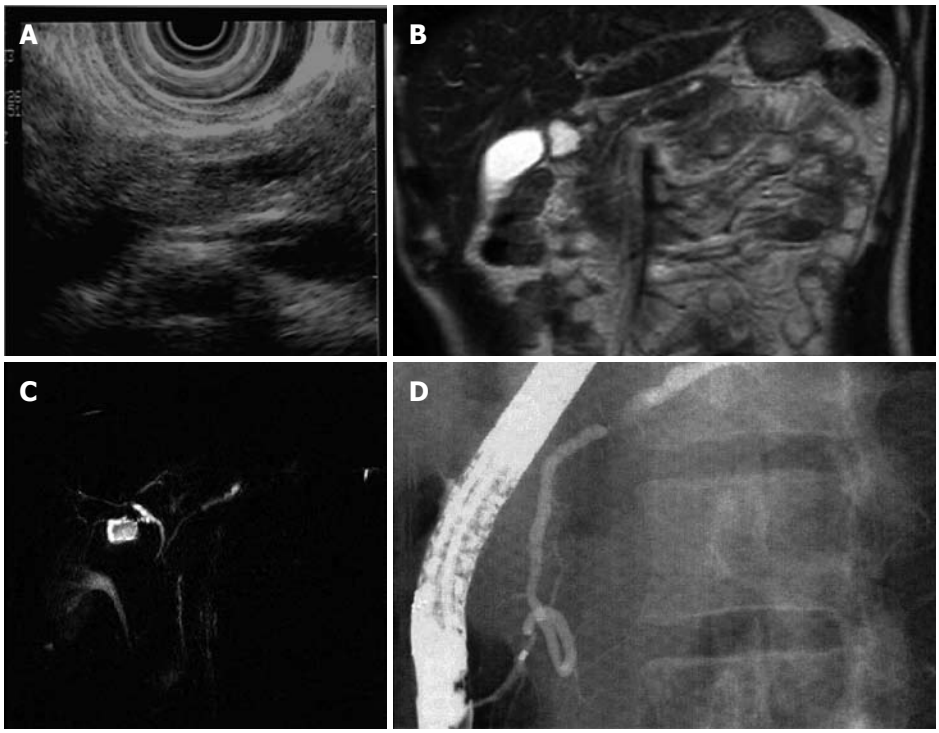

Figure 4 Dilation of the main pancreatic duct. Endoscopic ultrasonography was demonstrated presence of a small pancreatic tumor. A: Ultrasonography; B: Magnetic resonance imaging; C: Magnetic resonance cholangiopancreatography; D: Endoscopic retrograde pancreatography.

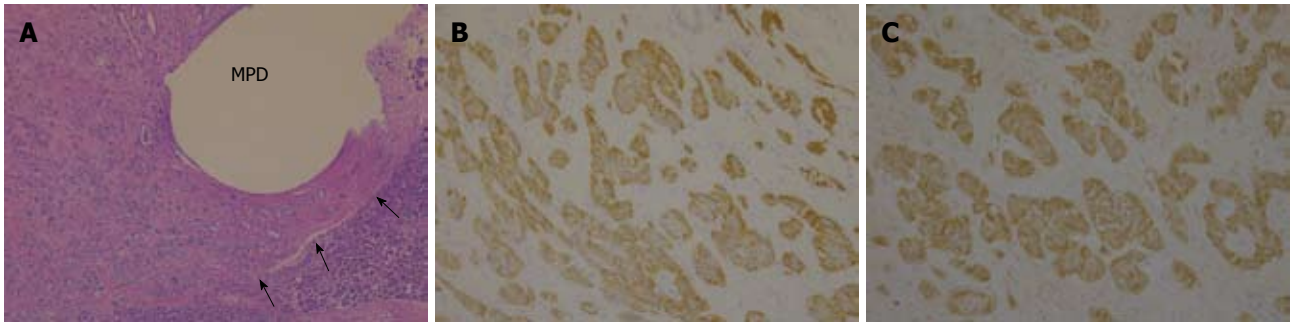

Figure 5 Pathological findings (hematoxylin and eosin staining) and immunohistochemical staining. A: A 4 mm × 5 mm tumor was detected (arrows); B: Chromogranin A; C: Serotonin. MPD: Main pancreatic duct.

the production of transforming growth factor β *via* selective activation of the 5-HT$_{2A}$ serotonin receptors[16]. Shi *et al*[11] reported that a small portion of pancreatic endocrine neoplasms produces serotonin. Serotonin production may be associated with stromal fibrosis, and fibrosis, in turn, can cause stenosis of the pancreatic duct. In clinical practice, imaging findings of pancreatic duct stenosis that is out of proportion to the size of the causative strongly enhanced mass or that does not have an associated distinct mass are indicative of a pancreatic neoplasm. Recently, it was reported that many pancreatic NETs express high levels of somatostatin receptors, so somatostatin-receptor scintigraphy (OctreoScan) can be imaged with a radiolabeled form of the somatostatin analog octreotide. Somatostatin receptor scintigraphy has proven particularly effective for visualizing gastrinomas, glucagonomas, and nonfunctioning pancreatic tumors. However, in another study of 37 patients with a NET, MRI and CT were substantially superior to SRS for detection of liver metastases. The sensitivity of OctreoScans was found to be particularly poor (< 35 percent) in lesions smaller than 1.5 cm in diameter[17].

In conclusion, we report 2 cases of serotonin-positive pancreatic endocrine tumors that caused stricture of the MPD in spite of the small size of the tumor. It is necessary to consider that the MPD stenosis and upstream dilatation caused by the tiny pancreatic endocrine tumor as the differential diagnosis in addition to chronic pancreatitis and pancreatic cancer.

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S- Editor Gou SX **L- Editor** A **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v18.i45.6674

World J Gastroenterol 2012 December 7; 18(45): 6674-6676
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Partial stent-in-stent placement of biliary metallic stents using a short double-balloon enteroscopy

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Received: May 14, 2012 Revised: July 27, 2012
Accepted: August 3, 2012
Published online: December 7, 2012

Abstract

Endoscopic intervention is less invasive than percutaneous or surgical approaches and should be considered the primary drainage procedure in most cases with obstructive jaundice. Recently, therapeutic endoscopic retrograde cholangiopancreatography (ERCP) using double-balloon enteroscopy (DBE) has been shown to be feasible and effective, even in patients with surgically altered anatomies. On the other hand, endoscopic partial stent-in-stent (PSIS) placement of self-expandable metallic stents (SEMSs) for malignant hilar biliary obstruction in conventional ERCP has also been shown to be feasible, safe and effective. We performed PSIS placement of SEMSs for malignant hilar biliary obstruction due to liver metastasis using a short DBE in a patient with Roux-en-Y anastomosis and achieved technical and clinical success. This procedure can result in quick relief from obstructive jaundice in a single session and with short-term hospitalization, even in patients with surgically altered anatomies.

Key words: Double-balloon enteroscopy; Malignant hilar biliary obstruction; Self-expandable metallic stent; Partial stent in stent; Roux-en-Y anastomosis

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Tsutsumi K, Kato H, Tomoda T, Matsumoto K, Sakakihara I, Yamamoto N, Noma Y, Sonoyama T, Okada H, Yamamoto K. Partial stent-in-stent placement of biliary metallic stents using a short double-balloon enteroscopy. *World J Gastroenterol* 2012; 18(45): 6674-6676 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i45/6674.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i45.6674

INTRODUCTION

Endoscopic intervention is less invasive than percutaneous or surgical approaches and should be considered the primary drainage procedure in most cases with obstructive jaundice. Recently therapeutic endoscopic retrograde cholangiopancreatography (ERCP) using double-balloon enteroscopy (DBE) has been shown to be safe and feasible, even in patients with surgically altered anatomies[1-4]. On the other hand, the placement of biliary stents is effective for the palliation of unresectable malignant hilar biliary obstruction in conventional ERCP[5-8]. In particular, as we previously described, endoscopic partial stent-in-stent (PSIS) placement of self-expandable metallic

stents (SEMSs) for malignant hilar biliary obstruction has been shown to be feasible, safe and effective[7,8], but it can be technically challenging. We report a case of a postoperative surgical patient who was managed successfully with a PSIS placement of SEMSs for malignant hilar biliary obstruction using a short DBE.

CASE REPORT

A 63-year-old male underwent total gastrectomy with Roux-en-Y reconstruction and sigmoidectomy due to simultaneous gastric and sigmoid colon cancer. Despite treatment with adjuvant chemotherapy, the patient's liver and lymph node metastases increased and caused obstructive jaundice, but no cholangitis. Computed tomography imaging showed dilation of the left intrahepatic bile duct due to liver metastasis, which occupied the right lobe (Figure 1A). For endoscopic biliary drainage, endoscopic retrograde cholangiography with a short DBE, EC-450BI5 (Fujifilm, Tokyo, Japan), was performed. The cholangiography revealed hilar biliary obstruction and a dilated left intrahepatic bile duct with tumor invasion extending to the bifurcation of the left lateral sectional bile duct branches (Figure 1B). After needle-knife sphincterotomy, a 0.035-inch guidewire was passed selectively into the left lateral superior bile duct branch (B3). The first uncovered SEMS (Zeostent 10 mm × 80 mm; Zeon Medical Inc., Tokyo, Japan) was deployed, with the proximal end in B3 and the distal end in the common bile duct. The guidewire remained in place, and the delivery system was removed. Subsequently, the wire was passed by catheter into the left lateral inferior bile duct branch (B2) through the mesh of the initial SEMS. Following balloon dilation (8 mm) at the stricture (Figure 2A), the second uncovered SEMS (Zeostent 10 mm × 100 mm) was smoothly deployed, with the proximal end in B2 through the mesh of the initial SEMS, forming a PSIS (Figure 2B). The patient was immediately relieved of jaundice and left our hospital in 7 d. He recovered enough to receive another round of chemotherapy on an outpatient basis.

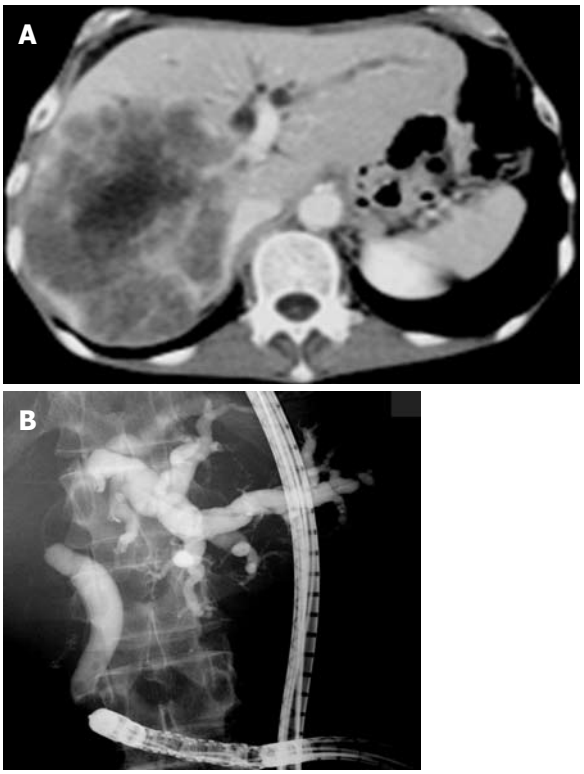

Figure 1 The hilar biliary obstruction due to liver metastasis occupying the right lobe and a dilated left intrahepatic bile duct with tumor invasion extending to the bifurcation of the lateral bile duct branch. A: Computed tomography image; B: Cholangiography.

DISCUSSION

In patients with surgically altered anatomy and long afferent limbs, ERCP by gastroenteroscopy, colonoscopy, or standard duodenoscopy is technically challenging and often unsuccessful because of an inability to reach the papilla or bilioenteric anastomosis. Recently, the use of a DBE or single-balloon enteroscopy has made therapeutic ERCP-including sphincterotomy, stone extraction, dilation of bilioenteric anastomotic stricture, and biliary stent placement-feasible and effective, even in patients with surgically altered anatomies[1-4].

According to a recent report on endoscopic intervention for the relief of malignant hilar biliary obstruction, the placement of SEMSs offers advantages over plastic endoprostheses in terms of stent patency and the number of reinterventions needed[5]. In addition, endoscopic PSIS placement of SEMSs for malignant hilar biliary obstruction has been shown to be feasible, safe and effective in conventional ERCP[5-8]. We previously reported that this procedure is safe and effective even in cancer patients receiving chemotherapy[8].

Therefore, in this case of a cancer patient with Roux-en-Y anastomosis, we used a short DBE to perform PSIS placement of SEMSs for malignant hilar biliary obstruction and achieved technical and clinical success. Almost all conventional accessories, including uncovered SEMS, were available, as we used a short DBE with a working channel of 2.8 mm in diameter and a 152 cm in length.

Percutaneous stent insertion for malignant obstructive jaundice had significantly higher 30-d mortality than the endoscopic method (33% *vs* 15%, $P = 0.016$) in a randomized trial[9]. Complications related with percutaneous transhepatic biliary drainage (PTBD), including intraperitrial hemorrhage, hemobilia, bile leakage, and pleural complications, can be avoided by using endoscopic drainage[10]. In our cases, 2 PTBD routes would have been required for the placement of 2 SEMSs at B2 and B3, respectively. In addition, 2 sessions would have been required for the placement of the SEMSs, that is, the SEMSs are usually placed one week after the initial PTBD. The endoscopic procedure could protect our patient from the risks associated with more invasive drainage procedures, such as PTBD and surgical drainage, the latter of which is associated with high morbidity and mortality rates. Furthermore, the patient needed no further long-term hospitalization

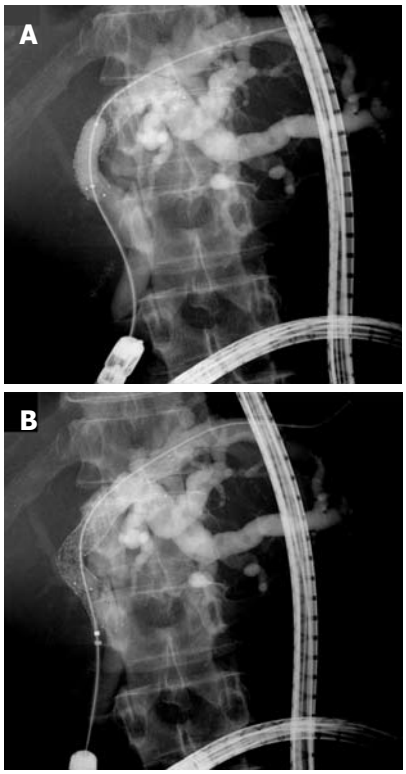

Figure 2 A partial stent-in-stent placement of biliary metallic stents using a short double-balloon enteroscopy. A: Following the placement of the first self-expandable metallic stent (SEMS), balloon dilation was performed at the stricture; B: The second SEMS was deployed through the mesh of the initial SEMS.

for the treatment of obstructive jaundice for the duration of his life. We think that this procedure is also indicated for patients in whom PTBD cannot be performed for various reasons, such as patients with severe coagulopathy, thrombocytopenia, a large amount of ascites, or an anatomically inaccessible location, e.g., patients with Chilaiditi syndrome.

In conclusion, endoscopic PSIS placement of SEMSs for the treatment of malignant hilar biliary obstruction using a short DBE was proved to be feasible and effective in a patient with Roux-en-Y anastomosis. This procedure can result in quick relief from obstructive jaundice in a single session and with short-term hospitalization, even in patients with surgically altered anatomies.

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S- Editor Gou SX **L- Editor** A **E- Editor** Xiong L

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v18.i45.6677

World J Gastroenterol 2012 December 7; 18(45): 6677-6681
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Plasmablastic lymphoma of the small intestine: Case report and literature review

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Received: July 5, 2012 Revised: August 28, 2012
Accepted: September 12, 2012
Published online: December 7, 2012

Abstract

Plasmablastic lymphoma (PBL) is a rare aggressive B-cell lymphoproliferative disorder, which has been characterized by the World Health Organization as a new entity. Although PBL is most commonly seen in the oral cavity of human immunodeficiency virus (HIV)-positive patients, it can also be seen in extra-oral sites in immunocompromised patients who are HIV-negative. Here we present a rare case of PBL of the small intestine in a 55-year-old HIV-negative male. Histopathological examination of the excisional lesion showed a large cell lymphoma with plasmacytic differentiation diffusely infiltrating the small intestine and involving the surrounding organs. The neoplastic cells were diffusely positive for CD79a, CD138 and CD10 and partly positive for CD38 and epithelial membrane antigen. Approximately 80% of the tumor cells were positive for Ki-67. A monoclonal rearrangement of the kappa light chain gene was demonstrated. The patient died approximately 1.5 mo after diagnosis in spite of receiving two courses of the CHOP chemotherapy regimen. In a review of the literature, this is the first case report of PBL with initial presentation in the small intestine without HIV and Epstein-Barr virus infection, and a history of hepatitis B virus infection and radiotherapy probably led to the iatrogenic immunocompromised state.

Key words: Plasmablastic lymphoma; Small intestine; Human immunodeficiency virus; Differential diagnosis

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Wang HW, Yang W, Sun JZ, Lu JY, Li M, Sun L. Plasmablastic lymphoma of the small intestine: Case report and literature review. *World J Gastroenterol* 2012; 18(45): 6677-6681 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i45/6677.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i45.6677

INTRODUCTION

Plasmablastic lymphoma (PBL) is a distinct, aggressive B-cell neoplasm that shows diffuse proliferation of large neoplastic cells resembling B-immunoblasts with an immunophenotype of plasma cells[1]. It was originally described in the oral cavity in the clinical setting of human immunodeficiency virus (HIV) infection, but may occur in other, predominately extra-oral sites, and these have been reflected in the current/revised 2008 World Health Organization classification[2]. Extra-oral PBL has been reported in various locations[3-6]; however PBL of

the small intestine was extremely rare. Here we present a rare case of PBL of the small intestine in an HIV-negative individual and review the literature.

CASE REPORT

A 55-year-old man presented with generalized abdominal pain, distension and vomiting for over a 1-mo period. He also described recent weight loss and anorexia. The above pathologic condition gradually became worse without relief of symptoms. Abdominal ultrasonography revealed multiple masses in the small intestine with extension to the bilateral adrenal glands. Radiographs and computed tomography (CT) scans of the chest, oral and peri-oral sites showed no abnormalities, and no peripheral lymphadenopathy was noted. Routine blood examination showed hemoglobin 106 g/L, white blood cell count 6.48 × 10^9 /L, neutrophils 64.4%, lymphocytes 15.3%, monocytes 10.2%. Serological testing demonstrated positivity for hepatitis B virus (HBV) surface antigen and HBV e-antigen, and negativity for HIV. The lactate dehydrogenase level (216 U/L) was in the normal range. Serum examination for Bence Jones' protein and rheumatoid factor were negative. The medical history was that he had a history of infection of HBV and underwent resection of squamous cell carcinoma in the maxillary sinus, followed by two courses of radiotherapy 7 mo previously.

During surgery, multiple tumor nodules were found in the intestinal wall and mesentery of the small intestine. Surrounding organs, including adrenal glands, liver, inferior vena cava and lumbar vertebrae were involved. Tumors were excised with adjacent portions of the small intestine and bilateral adrenal glands. A 55-cm long segment of the small bowel was obtained from the resection procedure. By gross examination, there was an irregular tumor nodule, volume 10 cm × 8 cm × 6 cm, in the intestinal wall surrounding the whole enteric cavity. Multiple tumor nodules ranging from 3.5 cm to 5.5 cm in diameter could be found in the adjacent mesentery. Tumor nodules in the left and right adrenal glands measured 8.5 cm × 8 cm × 4 cm and 8 cm × 5 cm × 2.5 cm respectively. The cut surface of these tumors was grey and soft. Selected tumor tissues were fixed in formalin and embedded in paraffin and cut into sections which were stained with the hematoxylin and eosin for routine histology. Additional sections of paraffin-embedded tissue were used for immunohistochemical staining and *in situ* hybridization analysis.

Histologically, the whole intestinal wall was diffusely infiltrated by malignant lymphocytes with abundant basophilic cytoplasm, eccentrically located pleomorphic nuclei, and single, centrally located prominent nucleoli (Figure 1A). The nuclei were round or oval or convoluted in shape and a large number of mitotic figures were apparent (Figure 1B). There were tumor emboli within the lymphatic vessels. Comprehensive necrosis of tumor cells was conspicuous. The tumor cells were scattered throughout the small intestine wall into the peripheral soft tissue and adrenal glands forming tumor nodules of different sizes. Furthermore, tumor cell proliferation in lymph node sinuses was conspicuous in the mesenteric lymph nodes.

On immunohistochemistry, the neoplastic cells were diffusely positive for CD79a (Figure 1C), CD138 (Figure 1D) and CD10 (Figure 1E), and partly positive for CD38 and epithelial membrane antigen. These cells were negative for CD45, CD20, CD3, CD30, ALK-1, Bcl-2, Bcl-6, Mum-1, CD56, HMB45, S-100, CD34, CD117 and cytokeratin. The cells showed kappa light chain restriction (Figure 1F). Nuclear proliferation rate, as assessed by Ki-67 staining, was approximately 80% (Figure 1G). Epstein-Barr virus (EBV) infection was not detected by *in situ* hybridization for EBV-encoded RNA (Figure 1H) or immunohistochemistry for EBV latent membrane protein-1. On the basis of these morphologic and immunohistochemical characteristics, the pathological diagnosis of PBL was made.

After surgery, the patient was treated with two courses of a standard CHOP (cyclophospamide, doxorubicin, vincristine, and prednisone) chemotherapy regimen. Unfortunately, during the second course of chemotherapy, repeat CT scans and ultrasonography revealed tumor progression with an increase in swollen cervical lymph nodes and abdominal tumor load. The patient died of multiple organ failure due to further deterioration 1.5 mo later.

DISCUSSION

As a rare entity of non-Hodgkin B cell lymphoma, PBL was first described as a specific clinicopathologic entity by Delecluse *et al*[7] as an aggressive B-cell lymphoma occurring in the oral cavity arising in the context of HIV infection. However, in recent years, several cases of PBL have been reported in patients without HIV infection, and several more have reported the occurrence of PBL in extra-oral sites, including the skin, subcutaneous tissue, stomach, anal mucosa or perianal area, lung, lymph node, and other regions[3-6,8,9]. The small intestine is a rare extra-oral site of involvement in PBL patients, and only two cases in HIV-infected patients have been reported previously[10,11]. In a review of the literature, this is the first case report of PBL with initial presentation in the small intestine and with multiple organ involvement in an HIV-negative individual.

As PBL is often associated with immunodeficiency, such as HIV infection, EBV plays an important role in the tumorigenesis of HIV-associated PBL. HIV infection creates a permissive environment for chronic EBV infection, with a subsequent latency that predisposes the EBV transformed B-cells to become malignant[12]. There is likely a connection between HIV-induced immunosuppression and the development of EBV-associated PBL[13]. However, recent investigation revealed that EBV infection was detected in only 17% of HIV-negative PBL cases, which suggest that this virus may not be a

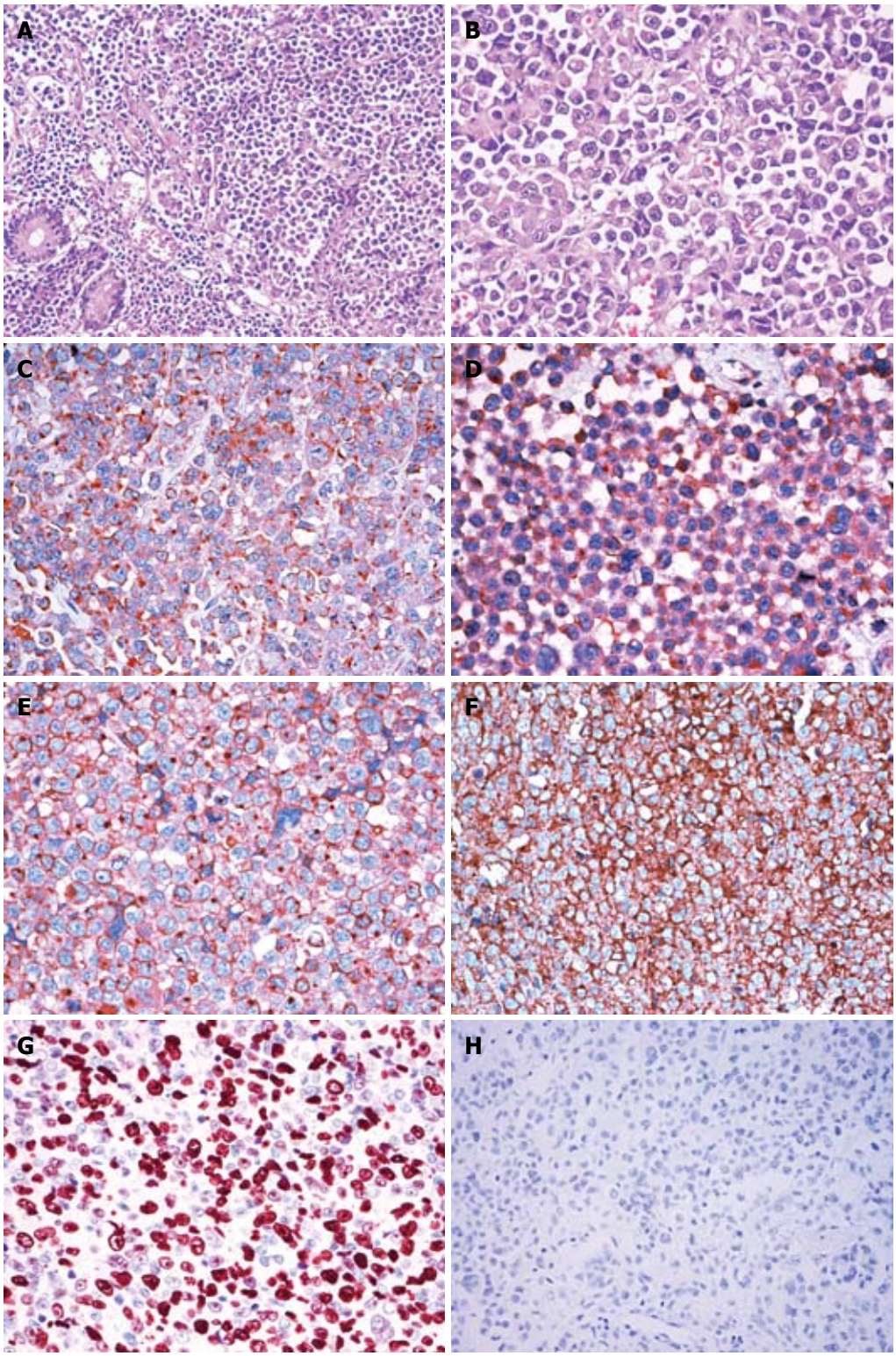

Figure 1 Plasmablastic lymphoma of the small intestine. A: Diffuse infiltration in the mucosa of the small intestinal by monotonous large atypical lymphoid cells [hematoxylin and eosin (H and E), original magnification ×200]; B: These atypical cells had a plasmablastic appearance, with abundant basophilic cytoplasm, eccentrically located pleomorphic nuclei, and single, centrally located prominent nucleoli (H and E, original magnification × 400); C-F: The atypical cells were diffusely positive for CD79a (C), CD138 (D), CD10 (E) and immunoglobulin light chain κ (F) (immunoperoxidase stain, original magnification × 400); G: Nuclear proliferation rate as assessed by Ki-67 staining was approximately 80% (immunoperoxidase stain, original magnification ×400); H: Epstein-Barr virus (EBV)-encoded RNA *in situ* hybridization for EBV shows negative staining in the nucleus of these atypical cells (original magnification ×400).

unique participant in the pathogenesis of PBL, especially in patients without HIV infection[14]. Cases of HIV-negative PBL have been mostly described after solid organ transplantation, in association with steroid therapy for autoimmune disease and some other types of immunosuppression[15,16]. The present patient was negative for HIV, and EBV infection was not detected by immunohistochemistry or *in situ* hybridization analysis, so an HBV infection and the history of radiotherapy probably led to the iatrogenic immunocompromised state.

The pathological differential diagnosis of PBL in the present case mainly included poorly differentiated primary or metastatic carcinoma, malignant melanoma, gastrointestinal stromal tumor (GIST), diffuse large B-cell lymphoma (DLBCL), Burkitt's lymphoma, anaplastic large cell lymphoma (ALCL), and plasmacytoma[3]. The characteristic morphology and immunophenotype of the tumor cells in conjunction with clinical features aid

Table 1 Differential diagnosis of plasmablastic lymphoma by immunohistochemistry

Marker	Current case	PBL	DLBCL	ALCL	BL	PC	GIST	Carcinoma	Melanoma
CD45	-	±	+	+	+	±	-	-	-
CD20	-	±	+	-	+	-	-	-	-
CD79a	+	±	+	-	+	+	-	-	-
CD38	+	+	-	-	-	+	-	-	-
CD138	+	+	-	-	-	+	-	-	-
CD3	-	-	-	±	-	-	-	-	-
CD30	-	-	±	+	-	-	-	-	-
CD10	+	±	±	-	+	-	-	-	-
ALK-1	-	-	±	±	-	-	-	-	-
Bcl-2	-	-	+	-	-	-	-	-	-
Mum-1	-	±	±	-	-	+	-	-	-
EMA	+	±	-	+	-	-	-	+	-
HMB45	-	-	-	-	-	-	-	-	+
S-100	-	-	-	-	-	-	-	-	+
CD34	-	-	-	-	-	-	+	-	-
CD117	-	-	-	-	-	-	+	-	-
Cytokeratin	-	-	-	-	-	-	-	+	-
LMP-1	-	±	±	-	-	-	-	-	-
EBER	-	±	±	-	±	-	-	±	-

PBL: Plasmablastic lymphoma; DLBCL: Diffuse large B cell lymphoma; ALCL: Anaplastic large cell lymphoma; BL: Burkitt's lymphoma; PC: Plasmacytoma; GIST: Gastrointestinal stromal tumor; +: Positive; -: Negative; ±: Variable expression.

in the differential diagnosis (Table 1). Poorly differentiated carcinoma may be differentiated from PBL according to its consistent immunological staining for cytokeratin. Malignant melanoma can be ruled out by using S-100 protein and HMB45. GIST is a common kind of mesenchymal tumor in the small intestine, usually characterized by expression of CD34 and CD117. In terms of negative expression for CD20 and morphological appearance, the present case of PBL can be clearly identified with other aggressive B-cell lymphomas, such as DLBCL and Burkitt's lymphoma. Histologically, the tumor resembled extranodal ALCL; however, tumor cells in ALCL are consistently immunoreactive for CD30 and usually immunoreactive for CD3 and ALK, which is different from our case. The differential diagnosis between PBL and poorly differentiated plasmacytoma is based mostly on clinical correlations, as both have similar morphological and phenotypic features[14]. The negative presence of serum monoclonal proteins and a high Ki-67/MIB-1 proliferation index help in the differential diagnosis from plasmacytoma in the present case.

In terms of clinical behavior, PBL is highly aggressive, with most of the patients dying in the first year after diagnosis. Most patients are at an advanced stage (Ⅲ or Ⅳ) at presentation[15]. HIV-positive and HIV-negative patients with PBL have different clinicopathological characteristics, including a better response to chemotherapy and longer survival in HIV-positive patients. HIV-negative patients had a median overall survival of 9 mo *vs* 14 mo in HIV-positive patients[13]. Furthermore, extra-oral PBL is more commonly disseminated (57% of the patients are at stage Ⅳ) at diagnosis. Meanwhile, the loss of CD20 associated with plasmacytic differentiation and very high Ki-67 index (> 90%) conveyed a worse prognosis[9]. In our case, the primary PBL of the small intestine had infiltrated multiple organs, accordingly a clinical stage Ⅳ was assigned. The HIV-negative state, extra-oral location, absent expression of CD20 and relatively high nuclear proliferation index in tumor cells collectively contributed to the more aggressive clinical course and worse outcome of this case.

In summary, we report a rare case of extra-oral PBL involving the small intestine in a HIV-negative patient, and describe the histologic and immunophenotypic findings. A diffuse infiltrative growth, rapid mitotic rate, and necrosis are consistent with the classification of PBL as a high-grade malignant lymphoma. Because PBL does not express the more common lymphoid and/or B-cell markers, it is easy to mistake them for a poorly differentiated carcinoma or sarcoma. Thus, diagnosis of PBL is challenging, particularly when it arises in extraoral locations and in immunocompetent patients. Recognition of this entity by the pathologist and clinician is important in establishing the correct diagnosis and treatment of the patients.

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S- Editor Lv S **L- Editor** Cant MR **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v18.i45.6682

World J Gastroenterol 2012 December 7; 18(45): 6682-6685
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Neuroendocrine carcinoma of the pancreas with soft tissue metastasis

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Received: August 17, 2012 Revised: October 26, 2012
Accepted: October 30, 2012
Published online: December 7, 2012

Abstract

Neuroendocrine carcinoma (NEC) of the pancreas is rare. We report the case of a 34-year-old man with pancreatic NEC with soft tissue metastasis. The patient presented with right upper abdominal discomfort. Computed tomography revealed a low-density heterogeneous mass in the tail and body of the pancreas that encroached on the greater curvature of the stomach and spleen. We performed exploratory laparotomy and total pancreatectomy with splenectomy and total gastrectomy. Histopathological analysis showed spindle-shaped cells with scanty cytoplasm and hyperchromatic nuclei, confirming a primary pancreatic NEC. One month after the surgery, the patient experienced leg swelling. Positron emission tomography-computed tomography revealed high uptake of fludeoxyglucose in the left leg, and the leg was amputated. Histopathological analysis confirmed metastasis of pancreatic NEC. The patient was followed up and received chemotherapy (etoposide and cisplatin). One month after amputation, the level of tumor marker neuron-specific enolase was 142.70 μg/L and computed tomography scan revealed an aggravated metastatic lesion. The patient suffered from unbearable pain and we treated him with odynolysis. Four months postoperatively, the patient died of respiratory failure.

Key words: Neuroendocrine carcinoma; Pancreas; Soft tissue metastasis; Neuron-specific enolase; Positron emission tomography-computed tomography

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Chen J, Zheng Q, Yang Z, Huang XY, Yuan Z, Tang J. Neuroendocrine carcinoma of the pancreas with soft tissue metastasis. *World J Gastroenterol* 2012; 18(45): 6682-6685 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i45/6682.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i45.6682

INTRODUCTION

Primary neuroendocrine carcinoma (NEC) of the pancreas is very rare, accounting for only 1%-1.4% of all pancreatic cancers[1,2]. Almost all NECs of the pancreas are discovered when the tumor is fairly large (mean: 6.2 cm, range: 2.5-20 cm) and it has metastasized to several distant organs such as the liver, adrenal gland, and brain, which explains the dismal prognosis[3]. We report a rare route of metastasis in this case.

CASE REPORT

We report a case of pancreatic NEC with soft tissue metastasis. The patient was a 34-year-old man who had no significant past medical history. He visited our hospital on January 1, 2012 with the symptom of right upper abdominal discomfort. A computed tomography (CT) scan revealed a low-density heterogeneous mass of 81

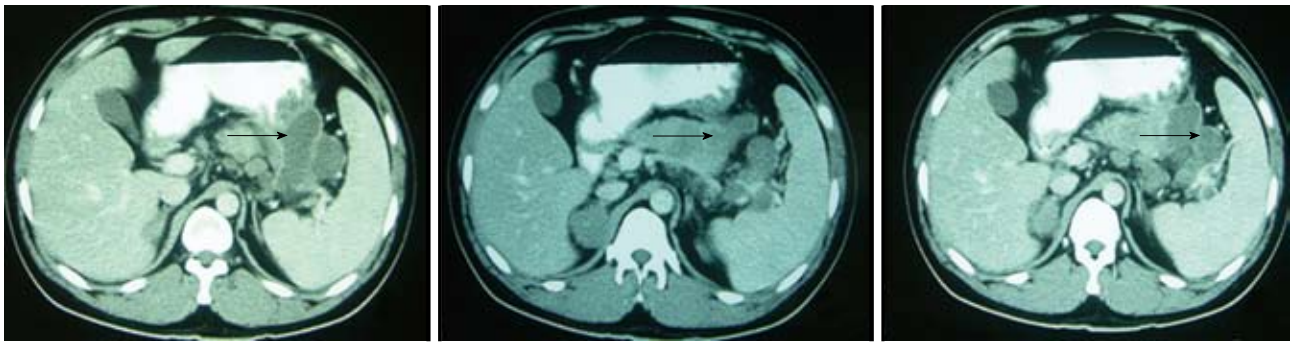

Figure 1 A computed tomography scan revealed a low-density heterogeneous mass of 81 mm × 68 mm in size in the tail of the pancreas (arrow) that invaded the greater curvature of the stomach and the spleen.

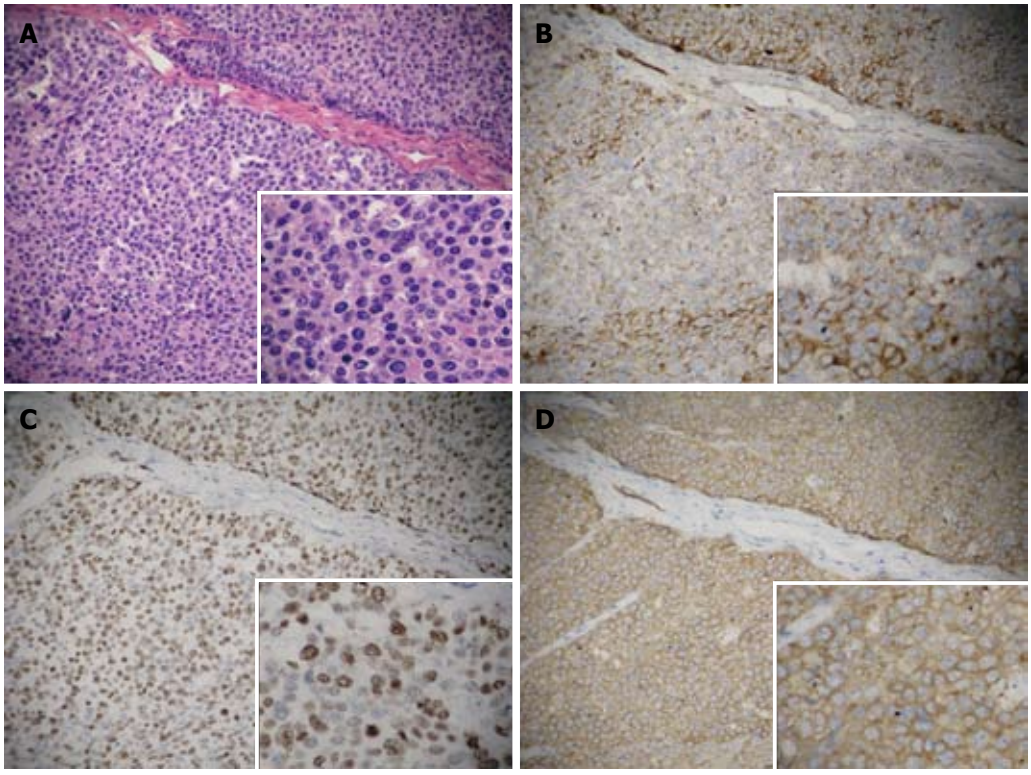

Figure 2 Pathology of the pancreas. A: Spindle-shaped cells with scanty cytoplasm and hyperchromatic nuclei (hematoxylin and eosin staining); B: Positively stained for chlorhexidine A; C: Approximately 80% of the tumor cells were positively stained for Ki67; D: Tumor cells were positively stained for synaptophysin (B, C and D: EnVision) (original magnification: ×200 and ×400).

mm × 68 mm in size in the tail and body of the pancreas that invaded the greater curvature of the stomach and the spleen (Figure 1). The laboratory findings were as follows: hemoglobin, 102 g/L; white blood cell count, 6.5 × 10^9/L; platelets, 374 × 10^9/L; aspartate aminotransferase, 18 U/L; alanine aminotransferase, 13 U/L; total bilirubin, 9.1 μmol/L; direct bilirubin, 4.0 μmol/L; serum creatinine, 59 μmol/L; carcinoembryonic antigen, 0.86 ng/mL (normal, < 10.0 ng/mL); alpha-fetoprotein, 2.37 ng/mL (normal, < 13.40 ng/mL); and carbohydrate antigen 19-9, 101.7 U/mL (normal, < 27 U/mL). The serum neuron-specific enolase (NSE) level was 59.94 μg/L (normal, < 17 μg/L). Chest X-ray examination revealed no signs of primary lung cancer or metastasis. In addition, there was no evidence of liver metastasis; therefore, exploratory laparotomy was performed. During the abdominal exploration, a 1-cm mass was detected in the head of the pancreas, and an 8-cm mass was detected in the pancreatic tail. The identified mass invaded the greater curvature of the stomach and the spleen. Consequently, we performed total pancreatectomy with splenectomy and total gastrectomy.

Histological examination revealed spindle-shaped cells with scanty cytoplasm and hyperchromatic nuclei. In addition, 9/12 lymph nodes were positive for metastasis. Hematoxylin and eosin staining (Figure 2) was performed on the paraffin-embedded sections. Immunohistochemical examination revealed chromogranin A and Ki-67 positivity.

One month after surgery, the patient exhibited leg swelling. Positron emission tomography-CT revealed high fludeoxyglucose uptake in the left leg and the relapse of carcinoma in both hila of the lungs (Figure 3). An orthopedist obtained a biopsy of the left leg, and the frozen section results indicated NEC. Therefore, the left leg of the patient was amputated below the knee. The

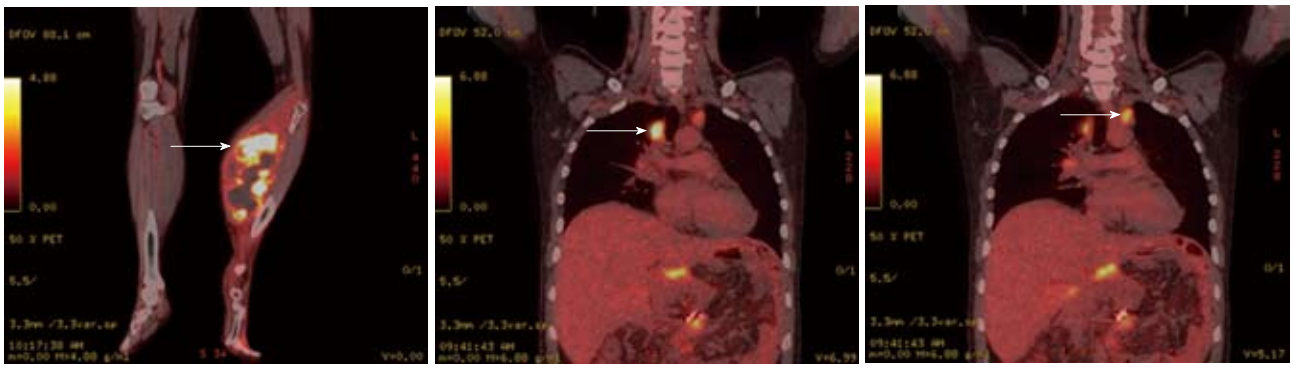

Figure 3 Positron emission tomography-computed tomography revealed high fludeoxyglucose uptake in the left leg and the relapse of carcinoma in both hila of the lungs.

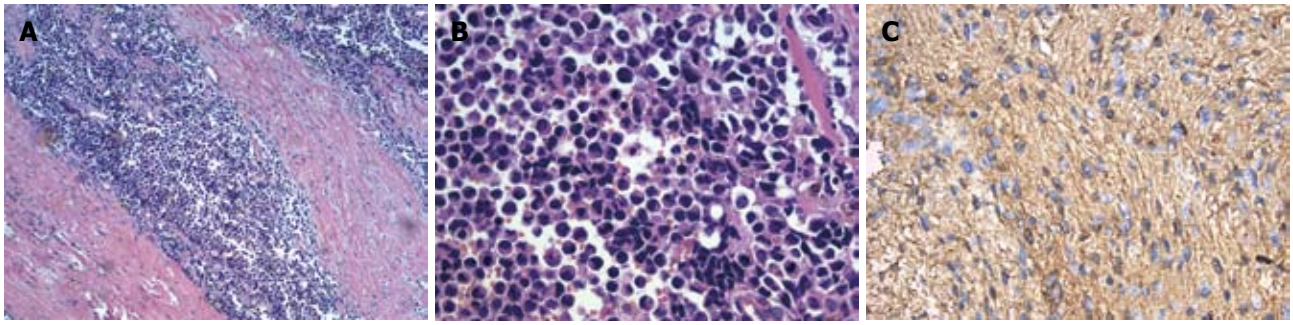

Figure 4 Pathology of the leg. A: Tumor cells displayed infiltrative growth in the striated muscle of the left leg [hematoxylin and eosin (HE) staining, original magnification: ×100]; B: Tumor cells were homogeneous (HE staining, original magnification: ×400); C: Tumor cells were positively stained for synaptophysin (EnVision, original magnification: ×400).

postoperative pathology of the left leg was metastatic NEC of the pancreas (Figure 4). The patient was followed up, and he received chemotherapy consisting of etoposide and cisplatin.

One month after amputation, the level of the tumor marker NSE was 142.70 μg/L, and a CT scan revealed an aggravated metastatic lesion. The patient reported unbearable pain, and he was treated by odynolysis. Four months postoperatively, he died of respiratory failure.

DISCUSSION

In a review of all published cases of pancreatic NEC, 91% of patients had metastases at the time of the initial diagnosis. According to the report by Vos *et al*[4], the most common sites of metastasis are the peripancreatic lymph nodes (62%), liver (38%), lungs (14%), bone marrow (14%), bone (10%), colon (10%), and adrenal gland (10%); rarer sites of metastasis include the spleen, gallbladder, kidneys, skin, and brain.

NSE can be considered a tumor marker that can be used in the diagnosis or assessment of treatment efficacy in patients with pancreatic NEC[5,6]. In our case, NSE was continuously aggravated.

Pancreatic NEC is a rare type of pancreatic cancer that has a poor prognosis[7]. The clinical course is typically aggressive, often characterized by disseminated disease at presentation and poor survival. In patients with extensive disease, a median survival as short as 2 mo has been reported, whereas in patients with limited disease, a median survival of up to 34 mo has been described[8]. Min Sung Chung reported a case of primary pancreatic NEC with unusually long-term survival after multimodal therapy. This patient remains in good health 36 mo after surgery[9]. In our case, the tumor extended beyond the pancreas with regional lymph node involvement. Because there was extension beyond the locoregional boundaries (extensive disease), we could perform palliative surgery. The patient in our case survived 4 mo on combined chemotherapy.

Some trials have revealed improved oncologic outcomes when patients are treated with regimens similar to those used for small cell cancers of the lungs[10]. The regimen of cisplatin, etoposide, and radiation is generally favored for pancreatic NEC[11]. This is the first reported case of pancreatic NEC with soft tissue metastasis. As mentioned previously, pancreatic NEC is a rare type of pancreatic cancer that has a poor prognosis. These adjuvant approaches should be considered in addition to surgery.

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S- Editor Gou SX **L- Editor** Logan S **E- Editor** Li JY

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v18.i45.6686

World J Gastroenterol 2012 December 7; 18(45): 6686-6689
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Sister Mary Joseph's nodule as a first sign of pancreatic cancer

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Author contributions: Bai XL and Liang TB designed the research; Zhang Q, Tang Y, Cao CH and Fu QH performed the research; Zhang Y and Gao SL discussed the clinical features of the disease; Bai XL, Zhang Q, Masood W and Masood N wrote the paper.

Supported by National Natural Science Funds for Distinguished Young Scholar, No. 30925033; National Natural Science Funds of China, No. 30801101 and No. 81171884; Innovation and High-Level Talent Training Program of Department of Health of Zhejiang
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Received: July 18, 2012 Revised: September 3, 2012
Accepted: September 12, 2012
Published online: December 7, 2012

Abstract

Sister Mary Joseph's nodule (SMJN) refers to a metastatic tumor of the umbilicus. It is a rare entity which arises from a malignancy in the intra-abdominal cavity. We herein describe a patient who presented with SMJN as his first sign of pancreatic cancer. It is an even more unusual case of SMJN. We therefore, suggest that pancreatic cancer should be included in the differential diagnosis when an umbilical mass is found. With the progress made in surgical procedures and other modalities, an early diagnosis will dramatically improve the prognosis of the patients.

Key words: Sister Mary Joseph's nodule; Pancreatic cancer; Umbilical metastasis; Diagnosis; Management

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Bai XL, Zhang Q, Masood W, Masood N, Tang Y, Cao CH, Fu QH, Zhang Y, Gao SL, Liang TB. Sister Mary Joseph's nodule as a first sign of pancreatic cancer. *World J Gastroenterol* 2012; 18(45): 6686-6689 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i45/6686.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i45.6686

INTRODUCTION

Umbilical tumors are rare and 30% of them are metastatic[1]. Sister Mary Joseph's nodule (SMJN) refers to a metastatic lesion of the umbilicus originating from intra-abdominal or pelvic malignant diseases. The most common primary sites of the metastasis are stomach, ovaries, colon, rectum and pancreas. Pancreas accounts for 7%-9% of the SMJN cases[2]. Almost 90% of the cases arise from the body and tail of the pancreas, but not the head[2].

We herein describe a male patient with pancreatic cancer who presented with SMJN as his first clinical sign. We also reviewed the published literatures on this disease from PubMed. Only 20 cases (including our case) of SMJN with pancreatic cancer as the first symptom and sign have been reported in English. The aim of this report is to provide new insight into the identification of pancreatic cancer presenting as SMJN.

CASE REPORT

The patient is a 40-year-old man who presented with

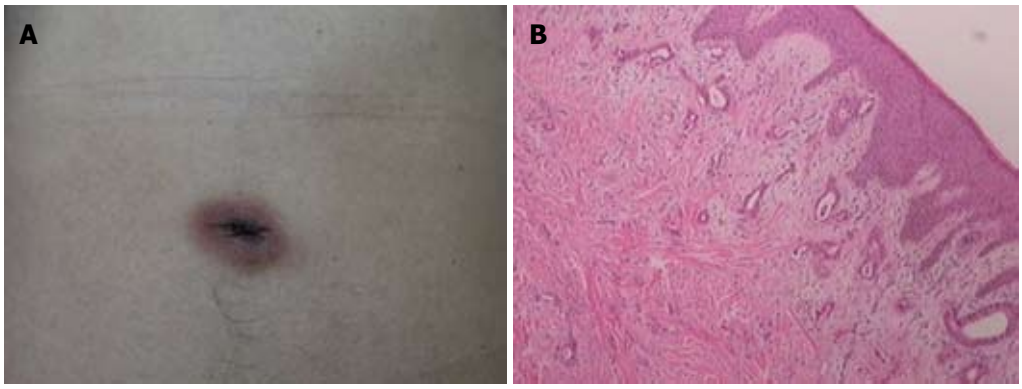

Figure 1 Appearance and pathology of umbilical tumor. A: A red nodule without ulcer appears in the umbilical region; B: Hematoxylin and eosin stain shows adenocarcinoma cell infiltration (×40).

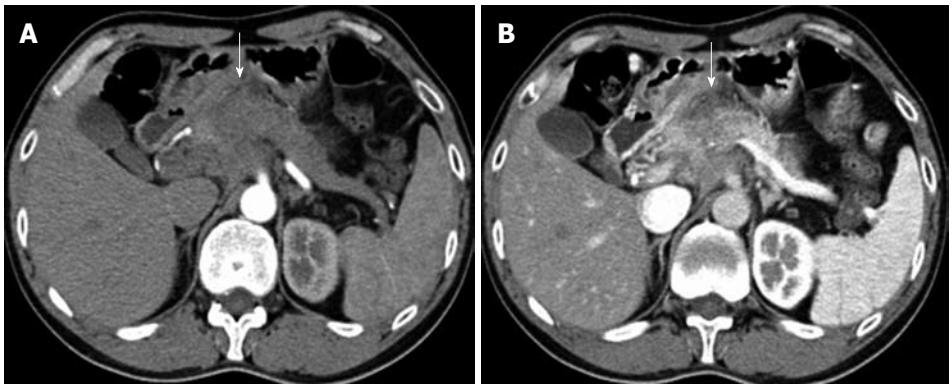

Figure 2 Abdominal contrast-enhanced multi-detector computed tomography scan. A: A mass in the neck of pancreas (arrow) is shown in arterial phase; B: The mass in venous phase.

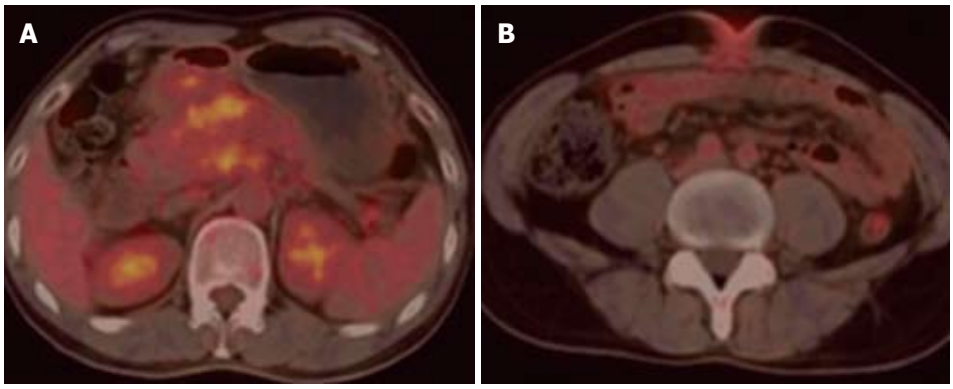

Figure 3 Positron emission tomography-computed tomography showed increased fludeoxyglucose uptake of tumors. A: Significant enhanced signal showing increased fludeoxyglucose (FDG) uptake at the site of pancreatic mass; B: Increased FDG uptake at the site of umbilical nodule.

swelling, redness and induration at umbilicus. The patient was first diagnosed with eczema at a local hospital and was treated without improvement. After six months, the patient consulted a dermatologist at our hospital. Physical examination showed an inflamed umbilicus with a nodule of 2 cm × 1.5 cm without ulcer (Figure 1A). An umbilical skin biopsy was taken and sent for pathologic examination. The pathology was suggestive of adenocarcinoma infiltrating dermis and epidermis (Figure 1B). Immunohistochemistry (IHC) showed cytokeratin (CK) (AE1/AE3) +++, carcinoembryonic antigen +++, and CK7 +. A metastatic adenocarcinoma was suspected based on all these findings.

Serum marker of carbohydrate antigen 19-9 (CA19-9) was elevated (202.1 U/mL). Other tumor markers were all in the normal range. An abdominal multi-detector computed tomography (MDCT) revealed a tumor at the neck of the pancreas with retroperitoneal lymph nodes enlargement as well as an umbilical soft tissue mass (Figure 2). An abdominal computed tomography (CT) angiography also showed involvement of retroperitoneal lymph nodes, superior mesenteric artery and vein, celiac trunk, proximal splenic artery, splenic vein and part of portal vein. A fluorodeoxyglucose (FDG) positron emission tomography-CT (PET-CT) scan demonstrated a mass located at the neck and body of pancreas with increased FDG uptake, with a maximal standardized uptake value (SUV) of 3.69, and thus malignancy was suspected. The umbilical mass with a maximal SUV of 2.85 was also detected and a metastatic malignancy was diagnosed by PET-CT (Figure 3).

Preoperative abdominal CT angiography showed the

tumor encasing celiac trunk and superior mesenteric artery, which, however, did not cause the occlusion or the stricture of theses arteries. According to our previous experience, the patient may still have a chance of resection. An exploratory laparotomy was then performed. Mild abdominal ascites was present. A hard mass of 8 cm at the neck and body of pancreas was discovered, with invasion of celiac trunk, superior mesenteric artery, superior mesenteric vein and portal vein. Several enlarged mesenteric lymph nodes and an umbilical mass of 2 cm × 1.5 cm as well as tiny diffused seeding nodules were observed. Diaphragmatic tumor invasion was visible. A biopsy was taken and a frozen section was sent for pathology. Adenocarcinoma infiltrating the striated diaphragmatic muscle tissues was confirmed. Surgical resection was abandoned because of distant metastasis of the cancer. The patient refused chemotherapy, and died four months later.

DISCUSSION

When a clinician observes an umbilical mass, his/her differential diagnosis should include both benign and malignant tumors. Benign tumors can be caused by a number of factors, including polyps, papilloma, myoma, fibroma, hemangioma, dermoid cyst, teratoma, pyogenic granuloma, omphalith, or endometriosis[1]. Malignant lesions account for 38% of all umbilical tumors, including both primary and secondary cancers[1]. These can be differentiated by means of biopsy collection and histopathological examination[3]. Cytology can not distinguish between primary and secondary malignancies[4].

The incidence of metastatic tumor originating from intra-abdominal malignancy to the umbilicus (SMJN) is very low. The most common primary sites of SMJN in men are stomach, followed by colon, rectum, pancreas and others[3]. In women, the most common origins are the ovaries, followed by stomach, colon, rectum, pancreas and others[2]. Pancreas is the fourth or fifth most common primary site of SMJN. The percentage of SMJN arising from pancreas ranges from 7% to 9%, among which SMJN presenting as the first sign of pancreatic cancer is even unusual[2,5]. To our knowledge after literature review, only 20 such cases, including our case, have been reported in English[6-11].

The metastatic routes of the pancreatic cancer to the umbilicus are thought to be by direct invasion from the peritoneum, through lymphatic or blood vessels and along embryonic remnants[6]. Generally, the prognosis of pancreatic cancer with SMJN is poor. The mean survival is 6-11 mo[6]. However, with early diagnosis and surgical intervention, a prolonged survival of 18 years was reported[6]. Currently, with the improvement of surgery, the resection rate of advanced pancreatic cancer has been increased. Moreover, with advances made in multidiscipline therapeutical modalities including chemotherapy, radiotherapy, targeted molecular therapy, immunotherapy, and others, the survival of the patients has also been improved.

It has been reported that resection of SMJN and the primary cancer of pancreas combined with other therapeutic modalities has improved the prognosis of the patients[9]. Therefore, an appropriate diagnostic algorithm is very important for early detection and rational treatment.

As mentioned above, for patients with umbilical nodules as the first clinical manifestation, biopsy should be performed first if the diagnosis is uncertain[3,4]. Almost all the cases of SMJN from pancreatic cancer reported in the literature are adenocarcinomas[4]. IHC staining for CK 7 and 19 are very important in diagnosing adenocarcinomas of pancreas[5]. Serum tumor markers such as CA19-9 should be examined and elevation of CA19-9 is considered as a strong evidence of the disease. Once SMJN deriving from pancreatic cancer is suspected, contrast-enhanced MDCT or magnetic resonance imaging (MRI) on pancreas should be done. Contrast-enhanced MDCT or MRI is sensitive enough to display the pancreas mass and its involvement in the adjacent organs and vessels. PET-CT is also recommended to detect other distant metastasis[12,13]. However, PET-CT is not sensitive to the identification of tiny seeding nodules in the intra-abdominal cavity. As in this case, PET-CT failed to display intra-abdominal diffusion.

An earlier diagnosis could have caught the tumor in its early phase, hence improving the prognosis of the patient. This paper is to remind physicians and surgeons of the importance of keeping in mind pancreatic cancer as one of their initial differential diagnosis when an umbilical nodule is presented. The recommended diagnostic algorithm is hopefully useful for the early detection of pancreatic cancer which appears as SMJN as its first sign.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v18.i45.6690

World J Gastroenterol 2012 December 7; 18(45): 6690-6692
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Malakoplakia of the esophagus caused by human papillomavirus infection

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Received: June 26, 2012 Revised: August 24, 2012
Accepted: August 25, 2012
Published online: December 7, 2012

Abstract

Malakoplakia is a rare granulomatous disease probably caused by infection and characterized histologically by Michaelis-Gutmann bodies. We report a more rarely seen case esophageal malakoplakia in a 54-year-old woman. She presented with coughing while eating and drinking. Gastroscopy showed yellow nodules in the esophagus, and endoscopic ultrasonography showed a space-occupying lesion in the substratum of the esophageal mucosa. All findings highly resembled esophageal cancer. Histopathological examination finally indentified this space-occupying lesion as malakoplakia and not cancer. Immunohistochemistry showed that she had human papillomavirus (HPV) infection in the esophagus, which indicates that infection was responsible for the malakoplakia. This is believed to be the first case of malakoplakia in the esophagus, and more importantly, we established that HPV infection was the initiator of esophageal malakoplakia.

Key words: Malakoplakia; Esophagus; Michaelis-Gutmann bodies; Human papillomavirus infection

Peer reviewer: Luigi Bonavina, Professor, Department of Surgery, IRCCS Policlinico San Donato, Piazza Malan, 20097 Milano, Italy

Yang YL, Xie YC, Li XL, Guo J, Sun T, Tang J. Malakoplakia of the esophagus caused by human papillomavirus infection. *World J Gastroenterol* 2012; 18(45): 6690-6692 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i45/6690.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i45.6690

INTRODUCTION

Malakoplakia is a rare granulomatous disease that was first described by Michaelis and Gutmann[1] in 1902, and is characterized by Michaelis-Gutmann bodies with cytoplasmic concentric laminated inclusions[2,3]. Malakoplakia most frequently involves the urinary tract, and less frequently, the gastrointestinal tract[4], and the esophagus is seldom involved. We report a case of esophageal malakoplakia in a 54-year-old woman.

CASE REPORT

Clinical findings

A 54-year-old woman was referred to the gastroenterology department with complaints of coughing while eating and drinking. Her past medical history included chronic atrophic gastritis, duodenitis and rheumatoid arthritis.

On clinical examination she was pale. Her chest X-ray, electrocardiogram, blood tests and serum α-fetoprotein results were normal. Gastroscopy showed soft yellow nodules in the right wall of the esophagus, which was 23.5-25.0 cm from the cutting tooth (Figure 1A). Endoscopic ultrasonography revealed a space-occupying lesion (8.1 mm × 5.1 mm) in the substratum of the esophageal mucosa (Figure 1B). ^{14}C-Urea breath tests were positive for *Helicobacter pylori* (*H. pylori*).

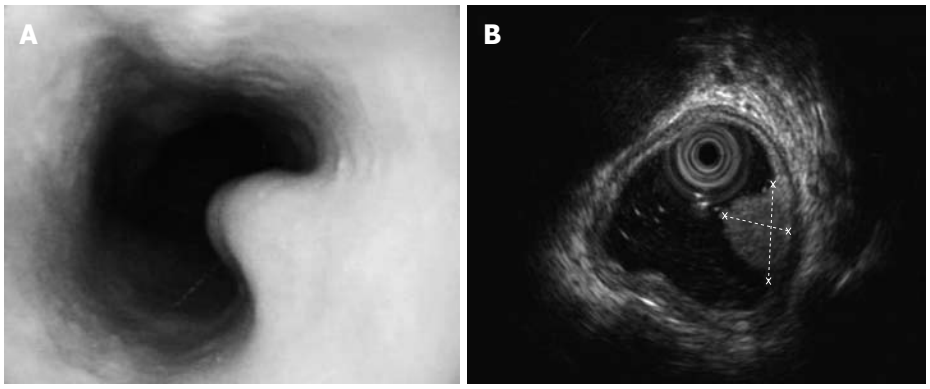

Figure 1 Soft yellow nodules protruding into the esophageal cavity. A: Gastroscopy presentation: soft yellow nodules protruding into the esophageal cavity; B: Endoscopic ultrasonography: a space-occupying lesion (8.1 mm × 5.1 mm) in the substratum sized 8.1 mm × 5.1 mm (dotted lines).

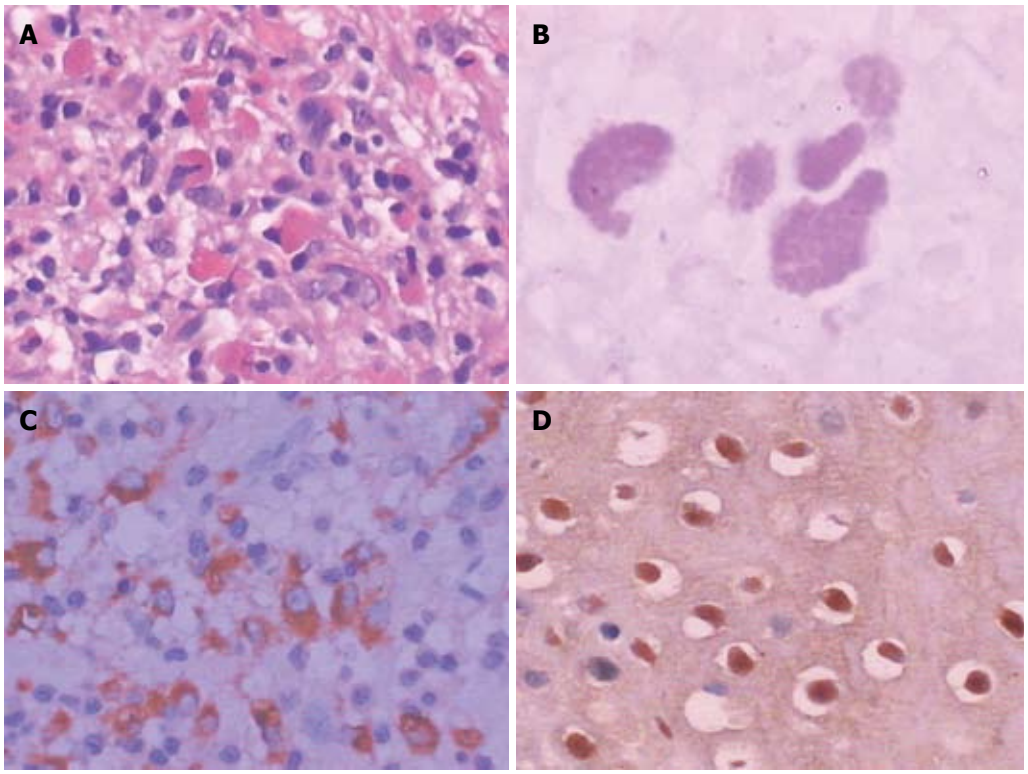

Figure 2 Michaelis-Gutmann bodies. A: Basophilic laminated inclusions in the cytoplasm (hematoxylin and eosin, ×200); B: Periodic acid-Schiff: targetoid appearance with a dense central core (×400); C: CD68 positivity by immunohistochemistry (×400); D: Esophageal squamous epithelium positive for human papillomavirus by immunohistochemistry, halo-shaped cells (×200).

Pathological findings

A local specimen of 1 cm × 1 cm was excision under gastroscopy for histopathological examination. Microscopic examination of hematoxylin and eosin stained sections showed a large amount of inflammatory cell infiltration in the substratum of the esophageal mucosa, mainly with lymphoid and histiocytic cells (Figure 2A). The presence of Michaelis-Gutmann bodies, with cytoplasmic concentric laminated inclusions of 5-15 mm confirmed the diagnosis. Follow-up examination supported this diagnosis. The Michaelis-Gutmann bodies were periodic acid-Schiff-positive (Figure 2B), and CD68-positive (Figure 2C) and human papillomavirus (HPV)-positive (Figure 2D) by immunohistochemistry, but negative for Eumycetes by hexamethylene diamine staining and *H. pylori* by Giemsa staining.

DISCUSSION

Malakoplakia is a chronic granulomatous inflammatory disease characterized by accumulation of granular basophilic Michaelis-Gutmann bodies. These bodies are generated from histiocytes, which are positive for CD68 antibodies, as well as positive for periodic acid-Schiff stain, and exhibit a targetoid appearance with a dense central core under light microscopy[4].

Malakoplakia has a worldwide distribution and does not have any racial, sex or age predilection[5]. Malakoplakia most commonly affects the urinary tract, as well as the gastrointestinal system, regional lymph nodes, skin, liver, and spleen[6-9].

The mechanism of malakoplakia is not well understood. Three postulates have been suggested. The first considers that microorganisms play a role in the pathogenesis. *Escherichia coli* infection is often found in the urinary tract[10], *Rhodococcus equii* in the lungs[11], and *H. pylori* in the stomach[12]. However, there have been no previous reports of microbial infection in the esophagus. In the present case, HPV infection was identified by immunohistochemistry. We consider that HPV infection plays an important

role in development of esophageal malakoplakia. Another possibility is that abnormal immune responses are involved in the pathogenesis[13]. Some immunosuppressive or chronic prolonged illnesses such as organ transplantation, acquired immunodeficiency syndrome, tuberculosis, sarcoidosis, and malignancy[14] can be associated with malakoplakia. The woman in this case report suffered from rheumatoid arthritis whose pathogenesis is considered to be related to an abnormal immune response, which we consider may also have been related to her malakoplakia. The third hypothesis is an abnormal macrophage response caused by defective lysosomal function. This results in macrophages being unable to digest fully the phagocytosed bacteria, accumulation of partially digested bacteria, and generation of Michaelis-Gutmann bodies.

Malakoplakia typical presents as irregular nodules or plaque, but it also exists as widespread mucosal multinodular or polypoid lesions, or large mass lesions under endoscopy. In the present case, it presented as endoscopic nodules.

The clinical appearance of malakoplakia varies from silent nodules to various different presentations according to the organ involved. In the urinary tract it presents with lower tract irritative symptoms such as frequency, dysuria and hematuria[15]. In the gastrointestinal system it can be clinically silent or can cause clinical symptoms such as diarrhea, abdominal pain, hemorrhage, or obstruction[16,17]. In the respiratory system it can appear as silent nodules that mimic bronchogenic carcinoma or tuberculosis[11]. Malakoplakia of the female genital tract usually presents with vaginal bleeding[18]. In the present case, the patient presented with coughing while eating and drinking, which resembled esophageal cancer.

Malakoplakia is generally considered a chronic, self-limiting inflammatory disease that may undergo spontaneous regression[19]. In the present case, despite the patient rejecting further treatment after receiving the pathological report, her symptoms disappeared and her condition did not develop. Follow-up endoscopic examinations 12 mo after resection revealed no changes in the patient's condition.

There are two therapeutic approaches to malakoplakia. Most cases have been successfully treated with antibiotics, for example, rifampicin, quinolone, and trimethoprim-sulfamethoxazole. The second approach is to attempt to correct the lysosomal defect by a cholinergic agonist, bethanechol chloride. Combination of antibiotic therapy and surgery provides satisfactory results. However, unnecessary radical surgical treatment should be avoided. The best choice depends on each specific patient. Our patient appeared to be cured by resection of the malakoplakia and showed no development during 1-year follow-up.

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
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World J Gastroenterol 2012 December 7; 18(45): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Acknowledgments to reviewers of *World Journal of Gastroenterology*

We acknowledge our sincere thanks to our reviewers. Many reviewers have contributed their expertise and time to the peer review, a critical process to ensure the quality of our World Series Journals. Both the editors of the journals and authors of the manuscripts submitted to the journals are grateful to the following reviewers for reviewing the articles (either published or rejected) over the past period of time.

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World J Gastroenterol 2012 December 7; 18(45): I
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Events Calendar 2012

January 13-15, 2012
Asian Pacific *Helicobacter pylori* Meeting 2012
Kuala Lumpur, Malaysia

January 19-21, 2012
American Society of Clinical Oncology 2012 Gastrointestinal Cancers Symposium
San Francisco, CA 3000, United States

January 19-21, 2012
2012 Gastrointestinal Cancers Symposium
San Francisco, CA 94103, United States

January 20-21, 2012
American Gastroenterological Association Clinical Congress of Gastroenterology and Hepatology
Miami Beach, FL 33141, United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts Oesophagus: Everything you need to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week 2012
Montreal, Canada

March 1-3, 2012
International Conference on Nutrition and Growth 2012
Paris, France

March 7-10, 2012
Society of American Gastrointestinal and Endoscopic Surgeons Annual Meeting
San Diego, CA 92121, United States

March 12-14, 2012
World Congress on Gastroenterology and Urology
Omaha, NE 68197, United States

March 17-20, 2012
Mayo Clinic Gastroenterology and Hepatology
Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

March 30-April 2, 2012
Mayo Clinic Gastroenterology and Hepatology
San Antonio, TX 78249, United States

March 31-April 1, 2012
27th Annual New Treatments in Chronic Liver Disease
San Diego, CA 92121, United States

April 8-10, 2012
9th International Symposium on Functional GI Disorders
Milwaukee, WI 53202, United States

April 13-15, 2012
Asian Oncology Summit 2012
Singapore, Singapore

April 15-17, 2012
European Multidisciplinary Colorectal Cancer Congress 2012
Prague, Czech

April 18-20, 2012
The International Liver Congress 2012
Barcelona, Spain

April 19-21, 2012
Internal Medicine 2012
New Orleans, LA 70166, United States

April 20-22, 2012
Diffuse Small Bowel and Liver Diseases
Melbourne, Australia

April 22-24, 2012
EUROSON 2012 EFSUMB Annual Meeting
Madrid, Spain

April 28, 2012
Issues in Pediatric Oncology
Kiev, Ukraine

May 3-5, 2012
9th Congress of The Jordanian Society of Gastroenterology
Amman, Jordan

May 7-10, 2012
Digestive Diseases Week
Chicago, IL 60601, United States

May 17-21, 2012
2012 ASCRS Annual Meeting-American Society of Colon and Rectal Surgeons
Hollywood, FL 1300, United States

May 18-19, 2012
Pancreas Club Meeting
San Diego, CA 92101, United States

May 18-23, 2012
SGNA: Society of Gastroenterology Nurses and Associates Annual Course
Phoenix, AZ 85001, United States

May 19-22, 2012
2012-Digestive Disease Week
San Diego, CA 92121, United States

June 2-6, 2012
American Society of Colon and Rectal Surgeons Annual Meeting
San Antonio, TX 78249, United States

June 18-21, 2012
Pancreatic Cancer: Progress and Challenges
Lake Tahoe, NV 89101, United States

July 25-26, 2012
PancreasFest 2012
Pittsburgh, PA 15260, United States

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OESO 11th World Conference
Como, Italy

September 6-8, 2012
2012 Joint International Neurogastroenterology and Motility Meeting
Bologna, Italy

September 7-9, 2012
The Viral Hepatitis Congress
Frankfurt, Germany

September 8-9, 2012
New Advances in Inflammatory Bowel Disease
La Jolla, CA 92093, United States

September 8-9, 2012
Florida Gastroenterologic Society 2012 Annual Meeting
Boca Raton, FL 33498, United States

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Current Problems of Gastroenterology and Abdominal Surgery
Kiev, Ukraine

September 20-22, 2012
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Prague, Czech

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Modern Technologies in Diagnosis and Treatment of Gastroenterological Patients
Dnepropetrovsk, Ukraine

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The Liver Meeting
San Francisco, CA 94101, United States

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Boston, MA 02298, United States

December 1-4, 2012
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Hollywood, FL 33028, United States

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Online Submissions: http://www.wjgnet.com/esps/
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World J Gastroenterol 2012 December 7; 18(45): I-VI
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

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Name of journal

World Journal of Gastroenterology

ISSN and EISSN
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

Editorial office
World Journal of Gastroenterology
Editorial Department: Room 903, Building D,
Ocean International Center,
No. 62 Dongsihuan Zhonglu,
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Indexed and abstracted in
Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifier, and Directory of Open Access Journals. ISI, Thomson Reuters, 2011 Impact Factor: 2.471 (32/74 Gastroenterology and Hepatology).

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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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

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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World Journal of Gastroenterology ®

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