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World Journal of Gastroenterology

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AIMS AND SCOPE

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 1353 experts in gastroenterology and hepatology from 68 countries.

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FRONTIER

Pancreatic enzyme replacement therapy for pancreatic exocrine insufficiency in the 21st century

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Abstract

Restitution of normal fat absorption in exocrine pancreatic insufficiency remains an elusive goal. Although many patients achieve satisfactory clinical results with enzyme therapy, few experience normalization of fat absorption, and many, if not most, will require individualized therapy. Increasing the quantity of lipase administered rarely eliminates steatorrhea but increases the cost of therapy. Enteric coated enzyme microbead formulations tend to separate from nutrients in the stomach precluding coordinated emptying of enzymes and nutrients. Unprotected enzymes mix well and empty with nutrients but are inactivated at pH 4 or below. We describe approaches for improving the results of enzyme therapy including changing to, or adding, a different product, adding non-enteric coated enzymes, (*e.g.*, giving unprotected enzymes at the start of the meal and acid-protected formulations later), use of antisecretory drugs and/or antacids, and changing the timing of enzyme administration. Because considerable lipid is emptied in the first postprandial hour, it is prudent to start therapy with enteric coated microbead prior to the meal so that some enzymes are available during that first hour. Patients with hyperacidity may benefit from adjuvant antisecretory therapy to reduce the duodenal acid load and possibly also sodium bicarbonate to prevent duodenal acidity. Comparative studies of clinical effectiveness of different formulations as well as the characteristics of dispersion, emptying, and dissolution of enteric-coated microspheres of different diameter and density are needed; many such studies have been completed but not yet made public. We discuss the history of pancreatic enzyme therapy and describe current use of modern preparations, approaches to overcoming unsatisfactory clinical responses, as well as studies needed to be able to provide reliably effective therapy.

Key words: Pancreatic insufficiency; Pancreatic enzyme replacement therapy; Lipase; Clinical trials; Steatorrhea; Fat malabsorption; Chronic pancreatitis

Core tip: In the last two decades, a number of studies comparing pancreatic enzymes and placebo have confirmed that pancreatic enzymes are superior to placebo for treatment of pancreatic malabsorption. While many patients achieved a satisfactory clinical response, individualization is often needed. Studies conclusively show that dose escalation is not a reliable method of obtaining further improvements and instead results in increased costs. Here, we describe alternate strategies for obtaining a satisfactory clinical response including changing to, or adding, a different product, adding non-enteric coated enzymes, use of antisecretory drugs and/or antacids, and changing the timing of enzyme administration.

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Dr. Graham is internationally recognized for his expertise in medicine and gastroenterology and is the author of more than 900 scientific papers, several books, and more than 100 chapters in medical text books. One of his papers is listed as one of the three most important papers in gastroenterology in the first 80 years of the *Annals of Internal Medicine*: (*i.e.*, Landmark Papers in Internal Medicine: The First 80 Years of Annals of Internal Medicine. Harold C Sox and Edward J Huth (Eds), 2009 (paper cited: Effect of treatment of *Helicobacter pylori* infection on the long-term recurrence of gastric or duodenal ulcer. A randomized, controlled study. *Ann Intern Med* 1992; 116: 705-8.).

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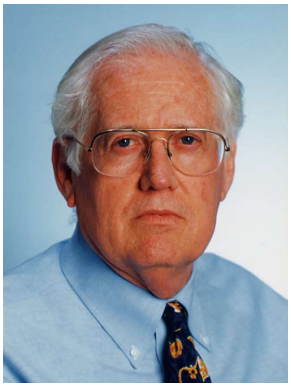

Figure 1 David Y Graham, MD, Professor, Department of Medicine, Michael E. DeBakey Veterans Affairs Medical Center and Baylor College of Medicine, 2002 Holcombe Blvd, Houston, TX 77030, United States.

INTRODUCTION

Orally administered pancreatic enzymes have been available since at least the 19th century, when many formulations were available as digestive aids. At that time it was known that orally administered enzymes were destroyed in gastric juice and that they were most effective when given in alkaline media[1]. A review of early 20th century research on the use of pancreatic enzymes for treatment of steatorrhea secondary to exocrine pancreatic insufficiency reported a wide variation in efficacy, yielding an overall 50% approximate reduction in steatorrhea[2]. The goal of pancreatic enzyme therapy is to restore normal fat absorption by delivering "a sufficient amount of active lipase at the right place, *i.e.*, duodenum and proximal jejunum, and at the right time, *i.e.*, in parallel with gastric emptying of nutrients"[3]. Achieving this goal has remained elusive despite the introduction and use of modern potent enzyme preparations[3-9].

Normal fat absorption requires integration of nutrient delivery with pancreatic and biliary secretions to accomplish hydrolysis and solubilization of ingested fats and fat-soluble dietary constituents. The normal process is finely tuned and requires coordination of many steps including controlled delivery of nutrients to the intestine, neutralization of acidic gastric contents, and secretion of pancreatic enzymes and bile to promote optimal digestion and solubilization of digestive products. These products of digestion then require a sufficient luminal intestinal surface area for absorption. Normally, the intestinal tract is able to process and absorb approximately 95% of ingested fat. There is considerable reserve capacity with all of the elements such that major anatomic alterations are required for weight loss surgery to be effective. The pancreas provides the bulk of the lipase needed for hydrolysis of triglycerides as well as bicarbonate to neutralize the acidic gastric contents. Pancreatic steatorrhea generally does not occur until lipase secretion is reduced by 90% or more[10].

Pancreatic steatorrhea is caused by disruptions of the normal process in which pancreatic enzymes are either inactivated or are otherwise unavailable (*e.g.*, blockage of the pancreatic duct, or resection or destruction of the glandular pancreas). Fungal, plant, and animal (especially porcine) pancreatic enzymes are available, and theoretically the simple addition of these enzymes with meals should resolve the deficiency and restore normal absorp-

Table 1 Reasons for a poor response to supplemental enzyme therapy

Inactivation of the enzymes in the stomach by acid and/or proteases
Inadequate mixing of the enzymes and nutrients during delivery to the small intestine such that a proportion of the meal is not exposed to appropriate concentrations of enzymes
Separation of enteric-coated microspheres from meal contents in the stomach
Low duodenal and small bowel pH fail to provide optimal conditions for lipase and bile salts to provide optimal digestion of the ingested nutrients
Delayed dissolution of enteric-coated enzyme microspheres in the small intestine
Incorrect or incomplete diagnosis

tion. Despite this hypothetical possibility, the administration of large doses of replacement pancreatic enzymes generally has not resulted in complete restoration of normal fat absorption[2,9,11-14].

One early approach was the use of enteric coating to protect the enzymes during passage through the stomach, but this was met with limited success[2,15]. Subsequent studies of normal gastric and pancreatic physiology identified many other barriers to successful treatment with pancreatic enzymes[16,17] (Table 1). This paper discusses the current status and clinical effectiveness of pancreatic enzyme therapy as well as possible approaches to overcoming the barriers to successful therapy. We also discuss the many myths and common misconceptions regarding therapy (Table 2). We begin with a historical review of the use of pancreatic enzyme therapy in the treatment of malabsorption due to chronic pancreatitis and cystic fibrosis; this historical perspective also provides the physiologic basis for the use of supplemental pancreatic enzymes and adjuvant therapies. We focus on overcoming the limitations of common strategies used to improve outcome, such as increasing the amount of lipase per meal, use of enteric-coating, the timing of enzyme administration in relation to meals, and use of antacids and antisecretory drug as adjuvant therapy. Success requires a strategy that is targeted to identify and overcome the specific barriers preventing correction of steatorrhea (Table 1). Currently, many patients achieve a satisfactory clinical response but few experience complete normalization of fat absorption; more than half often require individualized therapy to obtain symptomatic and nutritional relief[3-8].

The review is based on understanding the underlying physiology and the results of clinical trials in patients. It does not seek to comprehensively review all studies but rather to illustrate key principles and to show consistency of the results (typically failures to achieve correction of steatorrhea). Although meta-analyses have confirmed that enzyme therapy is superior to placebo, there is no evidence that one product is superior to another or that any will reliably eliminate steatorrhea. We also do not consider potential alternate indications for pancreatic enzymes such as abdominal pain in patients with chronic pancreatitis[18] or irritable bowel syndrome[19,20].

Table 2 Myths regarding modern microbead enzyme therapy

Currently available formulations will reliably correct steatorrhea
Increasing the dose of microbeads increases the effectiveness
Choice of dose depends on fat content of the diet
Proton pump therapy generally improves success with microbead therapy
Microbeads are fully protected in applesauce
Uncoated enzymes have no place in modern pancreatic enzyme therapy

MODERN ERA OF PANCREATIC ENZYME THERAPY

In 2004 the United States Food and Drug Administration (FDA) issued a requirement for manufacturers of prescription pancreatic enzyme products to submit new drug applications (NDAs) for all pancreatic enzyme products[21]. The FDA provided guidance on the minimal standards regarding the amount and stability of enzymes and the studies needed to establish efficacy (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm071651.pdf). The companies were told that only products receiving a new FDA approval would be allowed to remain on the market by 2008; this was later extended to 2010. The primary efficacy requirement was based on the comparison of the active product with placebo, which set a relatively low bar for efficacy. The FDA also requested, but did not require for approval, additional information about each product in terms of studies addressing gastric emptying, mixing, and dissolution time. The majority of products now available in the United States are enteric coated and formulated as microbeads, microtablets or microspheres (we use the terms "microbeads", "microtablets" and "microspheres" interchangeably). A non-enteric-coated product (Viokaze®, Forest Pharmaceuticals) was approved in 2012 (Table 3).

Most of the formulations are marketed in different strengths based on enzyme activity per capsule or tablet. Increasing the activity/dosage unit has generally been achieved by re-packaging the basic enzyme product into larger capsules, using different diameter enteric-coated beads, or both (Figure 2, Table 3).

The available prescription products are relatively expensive (Table 3). However, because "health food" stores still offer pancreatic enzymes as non-prescription "digestive aids" at a relatively low cost, many patients are likely to also use them. As noted, none of the currently available approved formulations have been shown to reliably achieve normal absorption irrespective of the quantity of lipase administered.

QUANTITY OF LIPASE REQUIRED TO ABOLISH STEATORRHEA

Normal pancreas

Normally, lipase is secreted early in the postprandial pe-

Table 3 Currently available United States Food and Drug Administration approved pancreatic enzyme preparations[1]

DRUG	Stregth Lipase USP	Preparation	Diagmeter\e	pH[1]	Cost per tablet (United States)	Cost per 1000 units
CREON®						
Creon 3000	3000	Capsule with enteric coated minimicrospheres	0.71-1.6 mm	5.5	$1.18	$0.39
Creon 6000	6000	Capsule with enteric coated minimicrospheres	0.71-1.6 mm	5.5	$1.30	$0.22
Creon 12000	12000	Capsule with enteric coated minimicrospheres	0.71-1.6 mm	5.5	$2.32	$0.19
Creon 24000	24000	Capsule with enteric coated minimicrospheres	0.71-1.6 mm	5.5	$4.56	$0.19
Creon 36000	36000	Capsule with enteric coated minimicrospheres	0.71-1.6 mm	5.5	$7.90	$0.22
Pancreaze®						
Pancreaze 4200	4200	Capsule with enteric coated microtablets	2 mm	5.5	$0.92	$0.22
Pancreaze 10500	10500	Capsule with enteric coated microtablets	2 mm	5.5	$2.29	$0.22
Pancreaze 16800	16800	Capsule with enteric coated microtablets	2 mm	5.5	$3.68	$0.22
Pancreaze 21000	21000	Capsule with enteric coated microtablets	2 mm	5.5	$4.58	$0.22
Zenpep®						
Zenpep 3000	3000	Capsule with enteric coated beads	1.8-1.9 mm	5.5	$1.27	$0.42
Zenpep 5000	5000	Capsule with enteric coated beads	1.8-1.9 mm	5.5	$1.21	$0.24
Zenpep 10000	10000	Capsule with enteric coated beads	2.2-2.5 mm	5.5	$2.39	$0.24
Zenpep 15000	15000	Capsule with enteric coated beads	2.2-2.5 mm	5.5	$3.47	$0.23
Zenpep 20000	20000	Capsule with enteric coated beads	2.2-2.5 mm	5.5	$4.71	$0.24
Zenpep 25000	25000	Capsule with enteric coated beads	2.2-2.5 mm	5.5	$5.83	$0.23
Ultresa®						
Ultresa 13800	13800	Capsule with enteric coated minitablet	2 mm	5.5	$3.01	$0.22
Ultresa 20700	20700	Capsule with enteric coated minitablet	2 mm	5.5	$4.46	$0.22
Ultresa 23000	23000	Capsule with enteric coated minitablet	2 mm	5.5	$5.47	$0.24
Pertyze®						
Pertyze 8000	8000	Capsule with bicarbonate buffered enteric coated microsphere	0.8-2.2 mm	5.5	$1.99	$0.25
Pertyze 16000	16000	Capsule with bicarbonate buffered enteric coated microsphere	0.8-2.2 mm	5.5	$3.99	$0.25
Viokase®						
Viokase 10440	10440	Non-enteric coated			$2.92	$0.28
Viokase 20800	20880	Non-enteric coated			$5.76	$0.28

[1]pH at or above which enzyme is designed to release most of the enzyme based on the package insert.

riod and reaches a maximum within the first hour; the majority of fat digestion and absorption normally occurs within the proximal small intestine[22]. The ability to measure lipase activity led investigators to ask whether there was a best, appropriate, or minimum amount of lipase needed to correct steatorrhea. The available data are confusing in part because lipase units are often presented in different units, making direct comparisons difficult. Many basic and clinical studies use either international units (IU) or United States Pharmacopeia (USP) units. Commercial products in the United States are rated in USP units (1 IU = 3 USP units). We will provide the results whenever possible in USP units. When the units are not clear (as in some older papers) we will simply state the units as lipase units or provide the units name used for that study. The strength of current products ranges from 3000 USP units to 36000 USP units of lipase per dosage unit (*e.g.*, per capsule) (corresponding to a range of 1000 to 12000 IU) (Table 3). The amount of postprandial lipase secreted under normal physiologic circumstances has been estimated at between 9000 to 18000 USP units/min[22,23] Measurements from a patient with a pancreatic fistula suggested that a 60 kg man would produce 192000 Cherry-Crandall units[24]. Overall, the results of such studies depend on the experimental methodology and may explain the wide variation noted[25]. As noted previously, the pancreas has a tremendous reserve capacity, and perfusion studies have suggested that approximately 5% of normal output is the threshold to maintain normal fat absorption[26]. Other studies report somewhat higher amounts[10,27].

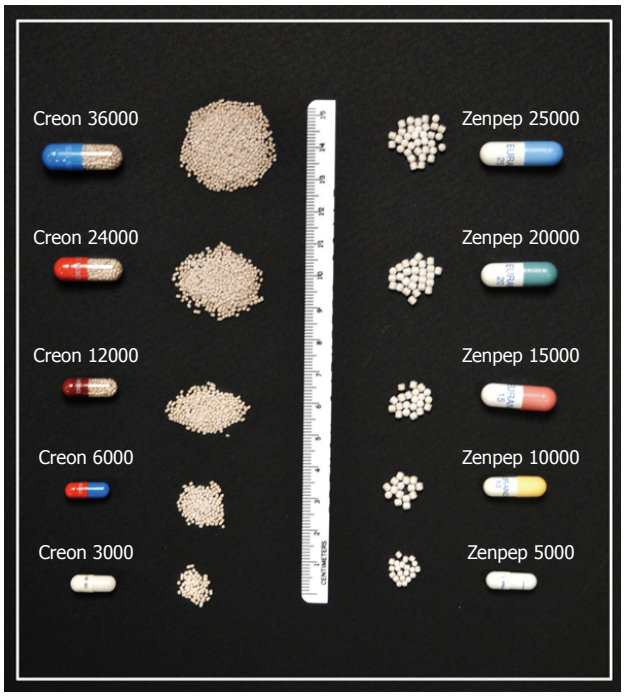

Figure 2 Pancreatic enzyme capsule size and contents increase as the pancreatic enzyme preparation dosage increases, suggesting that dose/unit increases are achieved by packaging the same basic pancreatic enzyme formulation into a larger capsule and/or larger beads.

Clinical results

Because it is difficult or even impossible to exactly simulate the normal integrated response of gastric emptying and pancreatobiliary secretion, estimates of the amount of lipase required to prevent steatorrhea are best determined clinically based on results of clinical trials. Trials using unprotected enzymes theoretically provide the most useful clinical measure, as they provide real time examples of pancreatic enzymes mixing and emptying with ingested nutrients coordinated with the function of the small intestine. However, interpretation of such studies is complicated by intragastric destruction of administered enzymes and by acidification of the duodenum, both of which can inactivate lipase and precipitate bile acids. Nonetheless, the available results probably provide our best estimates.

We performed studies with patients with varying degrees of acid secretory capacity and showed that we could abolish steatorrhea with approximately 30000 USP units of unprotected lipase given with meals (discussed in more detail in the section on the gastric pH barrier below). That study showed that a relatively small quantity of lipase was sufficient as long as the enzymes were able to mix with the meal and the lipase was not destroyed by gastric acidity (Figure 3)[28]. In a subsequent study with an enteric coated preparation, 2 of 6 patients experienced complete resolution of steatorrhea with only 18000 USP units of lipase with each meal when the enzyme was administered throughout the meal as enteric-coated microspheres (Figure 4)[29]. Overall, it seems reasonable to conclude that between 18000 and 30000 USP units of lipase per meal will result in resolution of steatorrhea, provided that lipase is delivered to the small intestine along with the nutrients and that low gastric and duodenal pH are not present. Achieving these coordinated events, however, to "deliver a sufficient amount of active lipase at the right place, *i.e.*, duodenum and proximal jejunum, and at the right time, *i.e.*, in parallel with gastric emptying of nutrients"[3] (Table 2) has proven difficult.

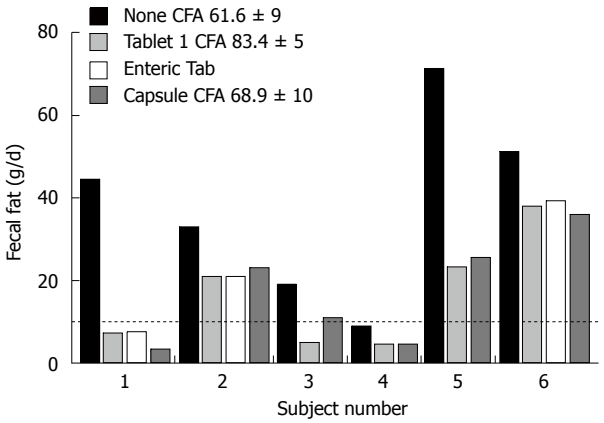

Figure 3 Results of different pancreatic enzyme preparations, tablets, enteric-coated tablets, and capsule in adults with exocrine pancreatic insufficiency. Approximately 30000 USP units of lipase were given with meals. Steatorrhea was corrected in those with low acid secretion. From[28] with permission.

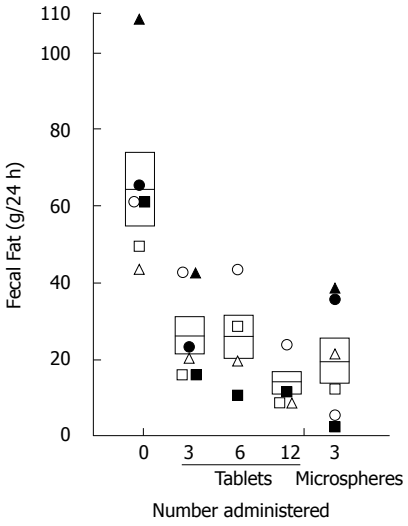

Figure 4 Effect of increasing the enzyme dosage on fecal fat excretion on a 100 gram fat diet. Enzymes were given 3 times per day with meals providing approximately 30000, 60000, or 120000 USP lipase units with each meal or as 18000 USP lipase units as enteric coated microspheres (*i.e.*, 3 tablets, 6 tablets or 12 tablets and 3 microsphere capsules with each meal). Each rectangle encloses the mean ± the standard deviation of the mean. The normal fecal fat is < 6 g/24 h. From[29] with permission.

Gastric pH barrier

Lipase is irreversibly inactivated at a pH of 4 or less. Trypsin and the other enzymes are more acid stable but

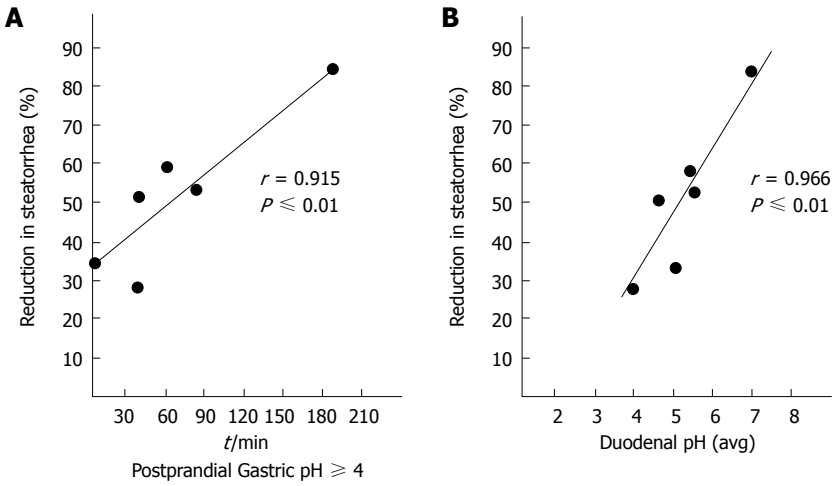

Figure 5 Correlation between percentage reduction in steatorrhea based on the median obtained with 30000 USP units of lipase given in tablets or capsules compared with the time of the postprandial gastric pH was > 4 (A) and in those same subjects compared with the mean post prandial duodenal pH (B). From[28] with permission.

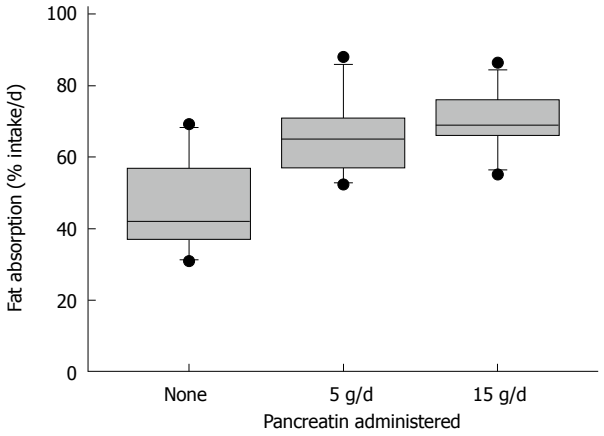

Figure 6 Results of a study comparing the response of enteric coated pancreatic enzyme in a cystic fibrosis patient population. Different doses of enteric coated pancreatic enzymes were taken four times daily immediately before meals and the corresponding average % fat absorption/d[11].

are also destroyed by pepsin in an acid environment[30,31]. Reliable enzyme therapy is therefore easiest to achieve in achlorhydric patients where the gastric pH barrier is absent. For example, we compared different enzyme formulations (2 tablet formulations and one capsule formulation produced by three different manufacturers, including one enteric coated tablet) in 6 patients who varied greatly in terms of their ability to produce acid[28]. The enteric coated tablet was effective only in one subject who also had hypo-/achlorhydria. We assessed the gastric barrier as the average time the gastric pH remained above 4 and the small intestinal pH barrier as the mean duodenal pH during meals. The effect of therapy on steatorrhea was almost identical for each individual subject (Figure 3) but varied between individuals with respect to gastric and duodenal acidity (*i.e.*, increasing acidity had a negative effect on reducing steatorrhea) (Figure 5)[28].

In subsequent studies with a different set of subjects, we examined whether the traditional approach of increasing the amount of unprotected enzymes would improve the effectiveness of therapy (in essence-was there a dose-response effect?)[29]. Doubling the amount of lipase from approximately 30000 USP units per meal to 60000 USP units per meal did not provide an improvement in fat malabsorption (Figure 4). However, quadrupling the lipase dose to 120000 USP (*i.e.*, 12 tablets per meal) did result in improvement in fat absorption (*i.e.,* decreased fat loss) but in only 2 of the 4 subjects tested (Figure 4). Importantly, none of these subjects had resolution of steatorrhea. As noted previously, in another study with different subjects, administration of only 18000 IU of lipase/day as an enteric-coated microbead preparation resulted in resolution of steatorrhea in 2 of the 6 subjects tested (Figure 4)[30].

As unprotected enzymes likely mix well with the nutrients, their effectiveness depends more on acid secretion and gastric emptying than on the quantity administered[30,32-34]. The window of effective unprotected enzyme therapy is defined as the time between ingestion and the time at which the gastric pH falls below 4 which inactivates lipase. Gastric contents tend to layer with the lowest pH being concentrated at the periphery of the meal. Thus, any lipase within the bulk meal may be protected and remain active, but will be inactivated upon mixing with acid contents in the antrum during emptying into the small intestine. Overall, our results confirmed longstanding clinical experience that, although increasing the amount of enzyme administrated may result in an improvement in fat absorption, it generally will not consistently eliminate steatorrhea (Figure 6)[11,12,29,35].

GASTRIC EMPTYING AS A BARRIER TO SUCCESSFUL PANCREATIC ENZYME THERAPY

The initial barrier is the acidic gastric environment that can inactivate pancreatic enzymes. The enzymes also must also mix with the nutrients to be delivered together to the duodenum. The normal gastric antrum grinds and returns food to the body of the stomach. Most nutrients are emptied as small particles (< 1 mm) suspended within

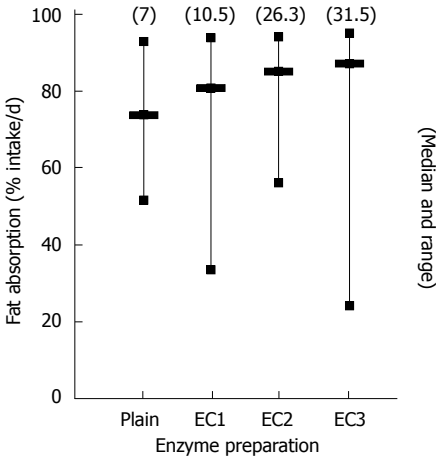

Figure 7 Randomized cross-over study in patients with cystic fibrosis and pancreatic insufficiency that compared plain uncoated enzymes (Pancrex V Forte *n* = 14) and 3 different enteric coated preparations [EC1:Pancreatin Merk, EC2: Creon and EC3: Pancrease (*n* = 19)] using the same lipase dosage. The median and range are shown of fecal fat absorption. With the numbers above the columns ( ) indicating the percent of patients with > 90% fat absorption. None reliably resulted in normalization of fat malabsorption[42].

the liquid layer[36]. Depending on their size and density, enzyme microspheres may separate from bulk nutrients and empty separately, thus impeding the interactions critical for digestion[37-39]. Normally, the stomach sieves and retains large particles until after the meal is emptied. This sieving occurs both in the proximal and distal stomach[36,37,40,41]. Currently available enteric coated enzyme beads vary with respect to enzyme content and diameter (*i.e.*, larger doses contain more units of enzyme per bead and may reach up to 2.5 mm in diameter) (Table 3). The dissolution and emptying characteristics of the different enzyme preparations and sizes remains unknown, as the FDA-requested studies have yet to be published. However, based on prior studies, each preparation is likely to have a different emptying profile. There is limited information available regarding the dispersion and emptying of enteric coated microspheres of different diameter and density, particularly in relation to fat malabsorption in humans. Comparative studies of 4 older preparations (Pancrex V Forte, Pancreatin Merk, Creon and Pancrease) showed differences in effectiveness, but it remains unknown whether the differences were primarily related to differences in the emptying of the beads or related to other factors (Figure 7)[42].

The ideal therapy is one that coordinates emptying of the meal and pancreatic enzymes. A significant proportion of ingested fat is emptied during the first hour of the meal, and normal physiologic lipase secretion is highest during this time[38,43-45]. However, enteric coated enzyme microbeads administered with meals tend to remain in the proximal stomach during the first hour, allowing a considerable proportion of fat to escape contact with enzymes and thus escape digestion[38,44]. Gastric emptying of enzymes and nutrients is better coordinated after the first hour, which is likely responsible for the improvement in absorption seen[38,44].

Overall, it is likely that a mismatch of emptying of fat and enzymes is a major contributor to the failure of currently available microbead preparations to fully correct steatorrhea. Bruno *et al*[39] administered microbeads before meals and noted that they separated from the meal and tended to clump in the antrum, although some of the beads emptied even prior to the meal. This finding suggests that one approach to improving therapy is to optimize the timing of the administration of microbeads to reduce or eliminate periods of dissociation of emptying of fat and microbeads.

Although the FDA requested that companies perform studies regarding kinetics of enzyme release of approved products (namely, the when, where, and how much enzyme is released), none of the studies performed to date have yet to be published (*e.g.*, clinicalTrials.gov NCT00676702, Pancrease MT, Johnson and Johnson Pharmaceutical, NJ, United States; NCT00744250, NCT00749099 Pancrecarb MS16, Digestive Care, PA, United States; NCT00559052, Viokase 16, Axcan Pharma, Canada). We requested this and other information such as the median and range of fat absorption from each manufacturer; however, the manufacturers were unresponsive. Importantly, no head to head comparative studies of current FDA approved products from different manufacturers or different formulations of a single product are available. It therefore remains unclear how much, if any, interchangeability there may be between or even within products. It is also not known whether the source of porcine pancreatic enzymes used by different manufactures comes from one or a number of sources.

SMALL INTESTINAL PH BARRIER

Normal lipid digestion and absorption involves hydrolysis of triglycerides as well as solubilization of the products of digestion for subsequent absorption[46,47]. These processes are pH dependent and are disrupted when pancreatic bicarbonate secretion fails to neutralize acidic gastric contents and prevent lipase inactivation and precipitation of glycine conjugated bile salts. In some patients this low pH environment extends far down the small intestine and impairs both digestion and solubilization[13,46,48]. In addition, enteric coated microbeads are designed to dissolve only when intraluminal pH is 5.5 or higher and may not dissolve until reaching the distal small intestine or even the colon[27,33,49-54].

USE OF ANTACIDS AND/OR ANTISECRETORY DRUGS TO EXTEND THE HIGH PH WINDOW

Successful use of unprotected enzymes requires the ability to prevent or reduce inactivation of administered lipase by gastric acid. Antacids have been used for this purpose since the 19th century. More recently the strategy

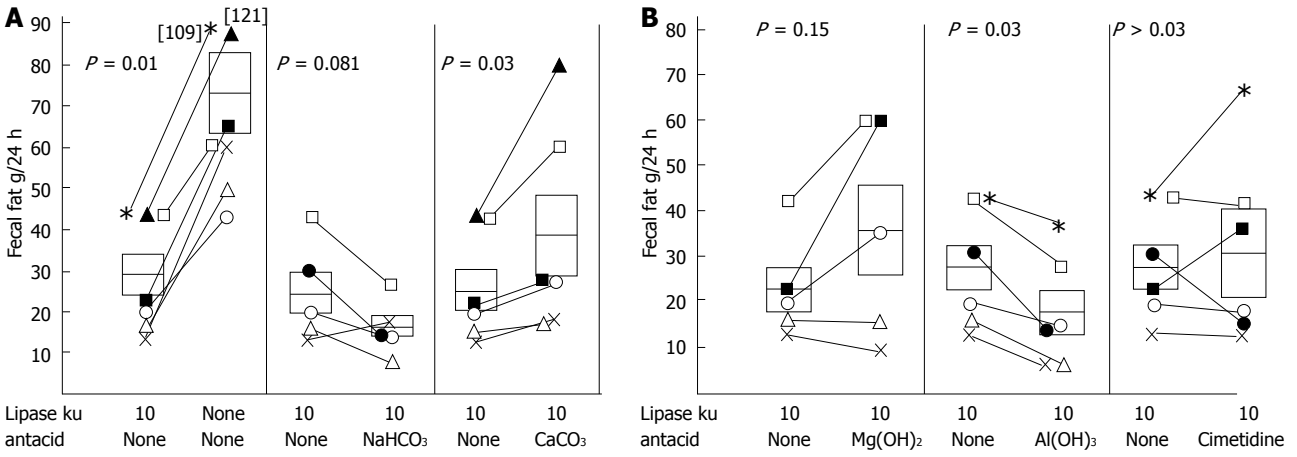

Figure 8 Effect of antacids and enzymes on the effectiveness of 30000 USP units of lipase per meal for the treatment of pancreatic steatorrhea. Each symbol represents a different patient. Sodium bicarbonate, magnesium aluminum hydroxide, aluminum hydroxide, or calcium carbonate were administered at the beginning and the termination of each meal. Cimetidine was given 30 min prior to the meal. From[58] with permission.

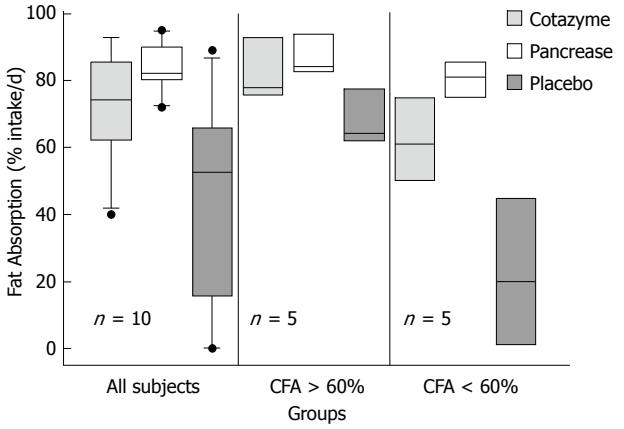

Figure 9 Randomized cross-over comparison of similar amounts of lipase administered as unprotected capsule (Cotazyme®) or enteric coated microspheres (Pancrease®) in cystic fibrosis patients with pancreatic insufficiency. Although the enteric coated preparation was better in those with the greatest degree of malabsorption, neither resulted in resolution of steatorrhea[61].

has shifted to antisecretory drugs; however, a combination of both may be the best option. The strategy to prevent inactivation of lipase differs from treatment of acid peptic disease. In peptic ulcer disease, the goal is to reduce gastric and duodenal acid load sufficiently to eliminate pain and heal the ulcer. In contrast, protection of lipase requires the much more stringent target that the gastric pH never fall to 4 or below (Table 2).

Early investigators reported only limited success in improving the effectiveness of enzyme therapy with co-administration of sodium bicarbonate or aluminum hydroxide[27,32,48,55-57]. We compared different antacids and the antisecretory drug cimetidine for their ability to improve the outcome of therapy with unprotected pancreatic enzymes[58]. We randomized subjects who had an incomplete response to 30000 USP units lipase per meal to receive commonly used doses of sodium bicarbonate (1.3 g; 12 mEq), aluminum hydroxide (30 mL; 57 mEq), magnesium-aluminum hydroxide (30 mL; 72 mEq), or calcium carbonate (1 g; 21 mEq). Each antacid was administered before and immediately after each meal (100 g fat per day)[58]. A final randomization was the 300 mg of the H_2-receptor antagonist, cimetidine, given 30 min before meals. Overall, cimetidine had no noticeable effect on fat absorption (Figure 8). In contrast, adjuvant therapy with either sodium bicarbonate or aluminum hydroxide resulted in a further reduction in steatorrhea (Figure 8). Strikingly, the highly effective antacids calcium carbonate and magnesium-aluminum hydroxide tended to reverse the beneficial effects of the enzyme therapy (Figure 8)[58]. Subsequent studies showed that the calcium and magnesium-containing antacids were effective in increasing intragastric and intraduodenal pH and improving the duodenal delivery of lipase and lipolysis[59]. However, both calcium and magnesium reacted with the fatty acids liberated to produce poorly soluble calcium and magnesium soaps that were poorly absorbed[59,60].

ENTERIC-COATING TO OVERCOME THE GASTRIC PH BARRIER

Using enteric coating is useful to bypass the gastric pH barrier and prevent gastric inactivation of pancreatic enzymes. The use of enteric coated microbead/spheres has resulted in more reliable results than had been obtained with enteric coated tablets (Figures 7 and 9)[42,61], but still fails to abolish steatorrhea for most patients[1,11,29,62-67]. The most common reasons given for an inadequate response to modern enteric coated enzyme therapy include: insufficient dosage, dissociation of the emptying of the microbeads and nutrients, premature opening of the microspheres in the stomach allowing intragastric destruction, long dissolution time which shifts the absorption sites distally, and rapid small intestinal transit which reduces mucosal contact time[33,36,37,43,44,51,68,69]. The benefits of modern enteric coated bead therapy appear greatest amongst those with the poorest responses to unprotected enzymes, most likely due to protection against rapid in-

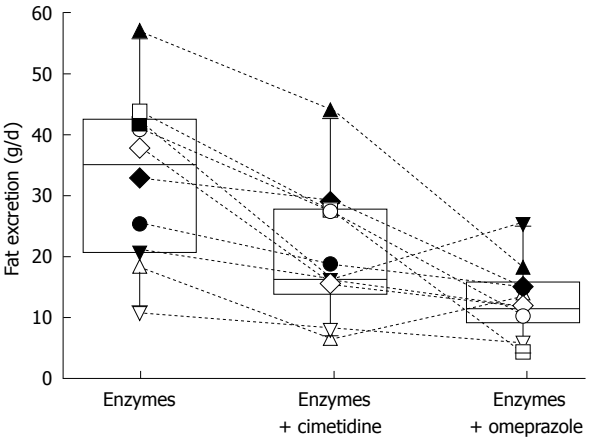

Figure 10 Box plot showing median and 25% and 75% and range for a randomized cross-over study comparing the effect of 1200 mg cimetidine or 60 mg of omeprazole on the effectiveness of pancreatic enzymes. Six tablets of unprotected enzymes (Cotazyme Forte® 36000 FIP units/meal) given 1/2 before meal and 1/2 during the meal). Both antisecretory agents improved outcome but neither reliably resolved steatorrhea. Data from[72].

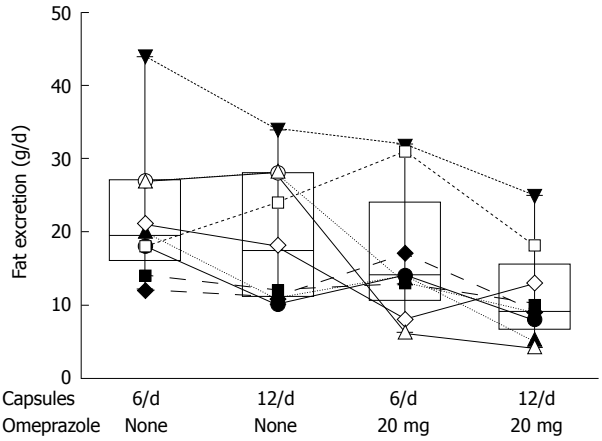

Figure 11 Box plot showing median and 25% to 75% range for a randomized cross-over study comparing the effect of doubling the dose of pancreatic enzyme microspheres (Pancrease®) and the effect of omeprazole in patients with cystic fibrosis and pancreatic insufficiency. Enzymes were taken 1/2 just before and 1/2 after meals. Omeprazole 20 min before breakfast[67].

tragastric inactivation of unprotected lipase[33,42,49,61,66,70,71].

Attempts to improving the efficacy of enteric coated microbead enzyme therapy

Few studies have provided sufficient details to develop hypotheses for testing or insights into why success or failure occurs. The Mayo clinic group tested an early enteric coated microsphere formulation with and without adjuvant acid suppressive therapy[34]. They found that of the 2 of the 6 patients had complete resolution of steatorrhea. Both these patients had high acid secretion and the intragastric pH remained below 5.5. The remaining 4 patients with incomplete responses had higher gastric pH, suggesting that the poor responders may have released the enzymes in the stomach where they were subsequently inactivated when the pH fell[34]. Bruno *et al*[72] compared adjuvant cimetidine or omeprazole with an enteric coated microsphere preparation (Cotazyme Forte®). Normal fat absorption was not observed, but they reported a progressive improvement with increasing suppression of acid secretion (Figure 10), suggesting that antisecretory drugs may be useful adjuvants. A possible mechanism is sufficient reduction of acid secretion to increase the duodenal and small intestinal pH and thus enhance dissolution and effectiveness of enteric coated microbeads[72]. Data to support this hypothesis comes from Regan *et al*[34] who showed that following cimetidine administration, the duodenal pH remained above 6 for up to 200 min postprandial.

The pH burden is related to emptying of acidic gastric contents into the duodenum, which can respond poorly because of abnormal duodenal/pancreatic bicarbonate secretion. Antisecretory drug therapy is potentially most useful in those with gastric acid hypersecretion to reduce the duodenal acid load and allow acid neutralization despite impaired pancreatic secretion of bicarbonate. In one study, Heijerman *et al*[67] compared different doses of enteric coated pancreatic enzymes with and without omeprazole in patients with pancreatic insufficiency due to cystic fibrosis with persistent steatorrhea. Increasing the dose of enzymes did not produce further improvement; however, increasing the enzyme dose and addition of omeprazole did (Figure 11). Overall, most studies with currently available preparations have not shown a consistent benefit for adding antisecretory therapy to enteric coated microbead therapy, except possibly among those with very poor response to enzyme therapy due to high gastric acid secretion[63,72-74]. Recent expert recommendations for use of pancreatic enzymes advise against the routine use of adjuvant proton pump inhibitor therapy[17].

Use of timing of dosing of pancreatic enzymes to improve outcome

In 1959, Jordan *et al*[12] compared 2 regimens in which 8 grams of unprotected enzymes (Viokase®) per day was given in 3 doses with meals or as 8 grams administered hourly from 8 a.m. to 7 p.m. (over 12 h). All 11 patients reduced their fecal fat excretion while taking pancreatic enzyme. Two patients failed to respond to the "with meals" regimen but experienced reductions in fat excretion with the hourly enzyme administration schedule. In contrast, Kalser *et al*[27] reported that administration of enzymes with meals (with adjuvant aluminum hydroxide) or on an hourly basis produced similar results. DiMagno *et al*[13] tested unprotected Viokase® (average of 10551 USP units lipase per tablet) administered either as eight tablets with each meal (2 tablets at the beginning, 4 tablets throughout the meal, followed by 2 tablets at the end of the meal) or as 2 tablets every hour for 4 doses at the onset of meal. In their study, irrespective of the dosing schedule, postprandial gastric pH fell below 4 after 40 min, the duodenal pH fell below 4 after 100 min, and less than 9% of lipase reached the duodenum.

Domínguez-Muñoz *et al*[73] performed a randomized three-way crossover study of 24 patients comparing

40000 USP units of Creon® enteric coated microbeads administered as 4 tablets before meals, 4 tablets just after meals, or 4 tablets throughout meals (as 1 before, 2 during, and 1 tablet after meals). Enzymes were administered only with the 3 main meals of the day given immediately before or after meals or given throughout the meal (as described above, with 10000 USP units before the meal, 20000 USP units during the meal and 10000 USP units after the meal). The authors used the 13C-mixed triglyceride breath test as a surrogate for fat absorption. The percentage of patients who normalized fat digestion was 50%, 54% and 63%, respectively. There were no statistically significant differences and no definitive conclusions can be drawn.

Other issues related to enteric coating

In 1905, Chase wrote that "it is a well-known fact that pancreatin in substance, solution, or simple tablet, is soon rendered inert by the gastric juice when taken into the stomach. The recognition of this fact has led to the manufacture of pills and tablets of pancreatin coated with keratin, salol, *etc*. While such coatings do protect the ferment from the action of gastric juice, it is a question if they are dissolved early enough in the intestine to allow the pancreatin to be of any service in digestion"[15]. The issues raised by Chase in his 1905 review remain unanswered more than 100 years later. Patients with pancreatic insufficiency have alterations in gastro-intestinal motility as well as a reduction in bicarbonate secretion resulting in low intestinal pH, and both of these mechanisms may lead to unpredictable transit and dissolution of the different products. Current formulations are designed to release the enzymes when the pH allows their survival. However, failure to achieve an adequate pH at which dissociation of the coating can occur may delay the site of dissolution to the distal small intestine or even the colon[33,51]. Guarner *et al*[68] compared duodenal and ileal enzyme content of normal controls and patients with pancreatic insufficiency. When normal patients and patients with pancreatic insufficiency received placebo, there was a gradient of higher lipase enzyme activity in the duodenum and lower activity in the ileum. When given enzyme therapy as 5 enteric coated capsules each containing 8000 FIP lipase units (total of 40000 FIP lipase units), the gradient was reversed.

Current enteric coated preparations are available as microspheres or microbeads whose dissolution rate was established using standard FDA-approved *in vitro* dissolution tests. However, little is known about their dissolution or potential differences in dissolution rate *in vivo*, especially at different pH and different luminal environments. Available products generally contain microbeads/spheres of uniform size within a specific dose. However between products and even among products at different doses, the beads may differ in shape, size, and surface area and all of these physical characteristics may affect the kinetics of release of the enzymes[75]. *In vitro* studies such as those described by Löhr *et al*[75] on previously available products would be welcome, especially if the results were directly compared to the results of *in vivo* studies. As noted previously, any data the pharmaceutical companies have has been withheld. Even when or if these data are provided, to be fully useful they must include comparison studies in the same patients to determine the effects of size, shape, differences in coating, or other factors on bioavailability. Such studies may require support by agencies dedicated to exploration of important scientific question without a vested interest that might result in withholding the results.

There are a number of considerations regarding evaluation of the dissolution characteristics of enteric coated enzymes. The rate of dissolution of the enteric coated beads at any particular pH would likely be an important measure in determining where the enzyme is delivered in the small intestine. Aloulou *et al*[51] evaluated the dissolution times in relation to pH of three preparations including the non-coated Eurobiol 12500 and 2 enteric coated preparations, Eurobiol 25000® and Creon 25000®. Uncoated Eurobiol 12500 had essentially instant bioavailability. The half dissolution time of Eurobiol 25000® at pH of 5.2 was 19.2 min, contrasting markedly with Creon 25000® whose half dissolution time at pH of 5.4 was 49.2 minutes. Importantly, this *in vitro* study did not take into account the effect of other confounders such the presence of bile and other substances normally present *in vivo*. Overall bioavailability is likely determined both by the threshold pH of dissociation as well as the rapidity of dissolution.

We tested the dissolution time on Creon 24000®, Zenpep 25000®, and Ultresa 23000® in informal studies using ileal fluid obtained from a patient with an ileostomy. One capsule of each enzyme preparation was placed a 15 mL conical tube containing 7 mL of ileal fluid obtained from a patient with an ileostomy and then centrifuged. The pH was adjusted to approximately 7.5. The experiment was done using a water bath at 38 Celsius. The test tube was manually inverted 3 times every 1.5 min and visually inspected for onset and time to complete dissolution of the capsule. pH was measured at each time interval (Table 4). Each experiment was done in duplicate. The results suggest there are likely differences in dissolution time among the different products and possibly between the same product as different size microbeads. Formal *in vitro* and *in vivo* comparisons are warranted.

Because clinical assessment is a notoriously imprecise measure of effectiveness, a simple, non-invasive measure of overall effectiveness is needed to allow comparisons between and among products[76]. The ^{13}C mixed triglyceride breath test currently appears to be the best option[77,78] as it provides dynamic data regarding gastric emptying, dissolution, and effectiveness of enzyme therapy. It has the added benefit of being simple, non-invasive, inexpensive, and allows for efficient repeated testing of the same subjects. Using a validated breath test allows hypothesis testing and rapid evaluation of different combinations such as timing administration of enzymes in relation to meals, effects of dosage, acid suppression, *etc*. These

Table 4 Dissolution time for pancreatic enzyme in ileal fluid

Pancreatic enzyme	Initial pH	Start to dissolve (min)	Completely dissolved (min)	
Creon® 24000	7.73 pH	9.0	45.8	7.28 pH
Ultresa® 23000	7.52 pH	10.5	30.0	7.48 pH
Zenpep® 25000	7.60 pH	15.0	33.0	7.59 pH

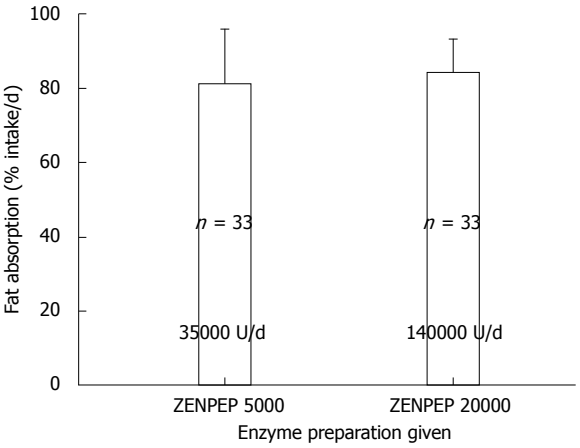

Figure 12 Effect of increasing the dose of enteric coated microbead therapy; seven 5000 USP unit tablets *vs* seven 20000 USP tablets (Zenpep®) on steatorrhea are shown (mean plus standard deviation). Increasing the dosage 4-fold resulted in no significant improvement in steatorrhea and did not result in correction of steatorrhea[4].

overall conclusions could then be tested in a traditional clinical trial. Breath testing also allows for easy and effective monitoring of therapy[77]. Unfortunately, despite being used in research for more than three decades, the test is not widely available outside of Europe and even there it is infrequently used.

APPROACHES TO THERAPY IN 2014-2015

Results with currently FDA approved enzyme preparations

The primary goal of enzyme therapy is to abolish steatorrhea. If this goal cannot be obtained, at the very least, one would like to achieve a coefficient of fat absorption >85% (*e.g.*, 15 g/d on a 100 g fat diet)[17,71]. The mean coefficient of fat absorption with modern enteric coated microspheres based on available data has typically been between 80% and 88% (*i.e.*, such that one third to more than one-half fail to achieve even this minimal desired outcome). Since at least the 19th century, the knee jerk response to inadequate results has been to increase the dosage. The "increase the dosage" strategy has carried over to the use of modern microbead therapy and the availability of high potency products[4,8,79] (Table 3). The published trials with currently available regimens were primarily designed to obtain regulatory approval for new products and for marketing purposes. The studies have therefore used similar protocols based on input from

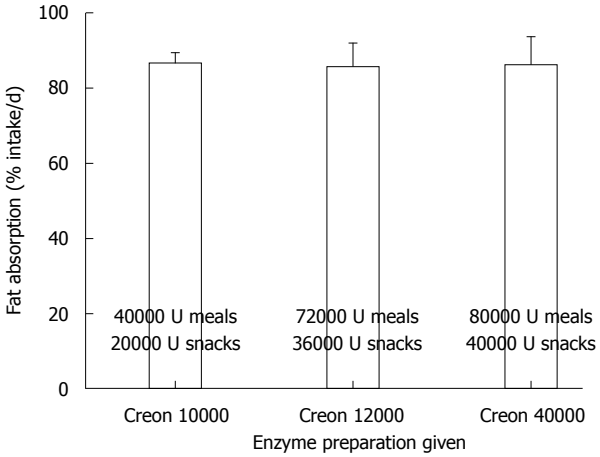

Figure 13 Summary data from 3 different randomized studies of different formulations of an enteric coated microbead product (Creon®). None of the formulations at the different doses given reliably resolved steatorrhea. Mean plus standard deviation of the different doses are shown[5,8,81].

the FDA (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm071651.pdf). These studies have been done well from a technical standpoint and used reliable methods for fecal collection and for analysis. The results are most often presented as the mean coefficient of fat absorption (CFA), which is calculated as [(fat intake - fat excretion)/fat intake] × 100 on a 72-h stool sample often collected in a controlled environment, plus the standard deviation. However, this presentation is of limited value to clinicians, as it does not provide definitive clinical data that would be useful in predicting clinical and symptom response, especially among patients with a previously unsatisfactory clinical response. For example, one would like to know the proportion of patients achieving a coefficient of fat absorption of at least 85%, as well as the median and range or 25%-75% values. Such data provide a clearer picture of what might be expected in clinical practice[42]. These data were requested from the manufacturers but not provided.

In some studies the patients may also not be representative. For example, Stern *et al*[80] included only patients who achieved at least 80% coefficient of fat absorption during a run-in phase on therapy, thus excluding the difficult to manage patients and improving the odds of an overall good outcome. In another study, approximately one-half of the subjects had minimal or no steatorrhea with placebo[4]. At least the data for the subgroup with significant steatorrhea was also provided separately in the outcome table[4]. Most trials have been relatively small because as they were powered only to detect a difference from placebo; however, the results may not extrapolate well to clinical practice. As shown in Figures 12 and 13[4,5,8,81] and Table 3, different formulations and lipase dosages have tended to provide similar results irrespective of the quantity of lipase administered. These results are consistent with the notion that only some of the lipase in the formulation was biologically available and overall

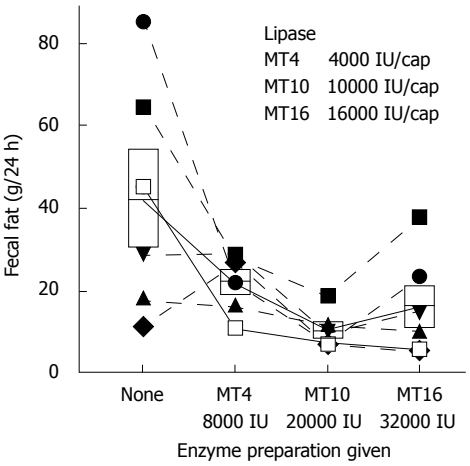

Figure 14 Effect of increasing the dosage of enteric coated microsphere preparation on fecal fat excretion is shown. Sorted by treatment groups and individual data for all subjects. Increasing the dosage from 8000 IU 4-fold (24000 to 128000 USP units) failed to show a clear dose response effect or to reliably resolve steatorrhea. The box shows the mean and standard deviation for each group. From[65] with permission.

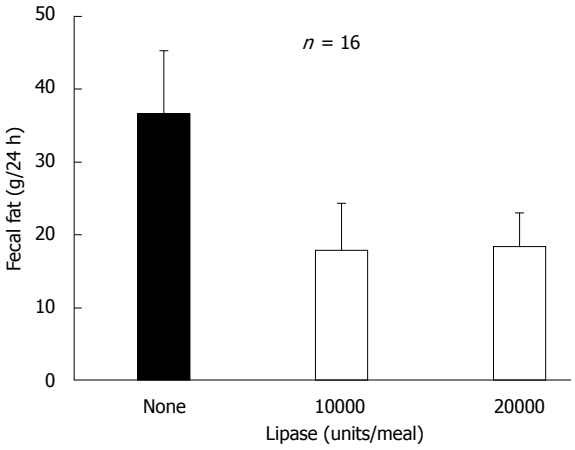

Figure 15 Effect of acid suppression with 60 mg of omeprazole on effectiveness of enzyme therapy with an enteric coated microsphere preparation (Pancrease®). Comparison of 2 dosing regimens 10000 (2 capsule of 5000 USP Pancrease®) or 20000 USP (4 capsule 5000 USP Pancrease®) lipase units per meal. The results were the same and neither resolved the steatorrhea[82].

was in excess of a threshold amount required to achieve the results reported. Importantly, these studies confirmed prior experience with enteric coated enzymes which also failed to show evidence of a dose response in terms of a reduction in steatorrhea[42,65,67,82] (Figures 7, 11, 14 and 15). Current products are priced in terms of dollars per units of enzyme (Table 3) such that the administration of more lipase than necessary serves only to increase cost to the patient without a corresponding increase in efficacy. A good example was a study that compared 7 capsules of Zenpep® 5000 (*i.e.*, 35000 USP units per day) a dose at which the authors expected "little or no effect on steatorrhea", with 7 capsules of Zenpep® 20000 (140000 USP units per day). The low and high doses produced similar outcomes (Figure 12)[4]. However, although the efficacy with high and low dose therapy did not differ, the cost of therapy per year was $11000 for high dose and $3000 for the equally effective low dose. These results confirmed that currently available products show (1) there is general lack of a dose-response effect; (2) increasing the dosage increases the cost more than the effectiveness; (3) a significant proportion of patients will still have clinically significant malabsorption despite enzyme therapy; and (4) a poor response to one dose generally signifies poor responsiveness to dose escalation.

One new preparation contains pancrelipase and sodium bicarbonate as a buffer to protect the enzymes and theoretically improve the pH in the small intestine (Pancrecarb®). It is called "highly buffered" although each capsule contains only 2.5 mEq of sodium bicarbonate. In clinical trials it was shown to be at best slightly better to not different from unbuffered capsules, and neither study achieved resolution of steatorrhea[83,84]. Currently, the FDA-approved Pertyze® is the only bicarbonate buffered pancreatic enzyme available. As noted above, studies of new concepts would probably be more efficiently initially evaluated using the ^{13}C-mixed triglyceride breath tests than through the use of expensive clinical trials.

Use of unprotected enzymes in the 21st century

An acid unprotected formulation of enzymes (Viokaze®) was recently FDA approved. While unprotected enzymes have limitations in relation to the relatively brief window in which the gastric pH is above 4, they may have a role in combination with enteric coated microbeads. In years past when *H. pylori*-associated atrophic gastritis was common, many adults had low acid secretion such that patients with pancreatic insufficiency often varied greatly in gastric secretory ability. In the modern era, *H. pylori* has become infrequent, and most adults exhibit normal acid secretion such that their intragastric pH falls to below 4 soon after eating and almost always within 60 min[54]. For these patients it is difficult to achieve or maintain an intragastric pH above 4 for a prolonged period using only antacids or antisecretory drugs. In the peptic ulcer era the goal of antacid or antisecretory therapy was to reduce acid output and thus the duodenal acid load. H_2-receptor antagonists typically reduce acid secretion by approximately 50%, which increases the average gastric pH for ulcer patients from approximately 1.4 to approximately 2, but increases the duodenal pH to above 4. Standard doses of proton pump inhibitors (*e.g.*, 20 mg of omeprazole) produce approximately a 90% reduction in acid secretion and an intragastric pH of 3 to 4[85]. A double dose (*e.g.*, 40 mg of omeprazole) provides 99% inhibition of acid secretion with narrow confidence intervals but will not reliably maintain the pH at 6 or above (which is the rationale for continuous infusion proton pump therapy in treatment of upper gastrointestinal ulcer bleeding)[85].

Studies of intragastric pH during meals have shown that the intragastric pH rapidly increases to the approximate pH of the meal, typically about pH 5, which stimulates the stomach to secrete acid maximally[54]. Initially, se-

creted acid is largely consumed by the buffering capacity of the meal such that average volume in the stomach remains relatively constant despite emptying. By 1 h, the intragastric pH falls to approximately 3, resulting in down-regulation of acid secretion allowing gastric emptying to exceed secretion such that the intragastric volume and the pH to continue to fall[86-91]. In normal subjects, one can expect the intragastric pH to fall below the threshold for lipase destruction between 30 min and one hour after eating. The longer the acid secretory rate is suppressed, the longer the lipase can remain active. In peptic ulcer disease, the recommendation was to administer antacids 1 and 3 h after meals in order to reconstitute the buffering capacity of the meal and achieve the maximum benefits for treatment of peptic ulcer disease. When used as an adjuvant to enzyme therapy, the goal is to maintain the pH above 4 or above for as long as possible in order to prevent inactivation of lipase.

pH is measured on a log scale such that each unit of change signifies a 10-fold change in acid concentration. Thus, a pH of 1 is equal to 100 mEq/L and a pH 6 equals 0.001 mEq/L. Parietal cells secrete acid at a high concentration (*e.g.,* 140-160 mEq/L); hence only a few active parietal cells secreting a small amount of concentrated acid can drop the pH below 4 and inactivate lipase[85]. Since high intragastric pH stimulates the stomach to secrete maximally, it is practically impossible to provide sufficient sodium bicarbonate or aluminum hydroxide to reliably maintain the intragastric pH above 5. However, the combination of an antisecretory drug to inhibit parietal secretion, coupled with an antacid to increase the pH and neutralize the small amount of acid secreted after inhibition of the majority of parietal cells, should be effective. Sodium bicarbonate is probably the ideal antacid as it is "natural," widely available in 325 mg (4 mEq) and 650 mg (8 mEq) tablets, and cheap. Although the ideal strategy remains to be determined experimentally, we recommend use of a proton pump inhibitor such as 40 mg of omeprazole daily along with 650 mg sodium bicarbonate tablets administered whenever unprotected enzymes are administered (*i.e.*, 1 tablet 2 or 3 times with the enzymes during the meal) and 1 and 2 h after meals. Current technology using the Smart Pill®[92] or Bravo®[93] to measure pH in the stomach and duodenum should rapidly identify the ideal timing and dosage of administration of the sodium bicarbonate.

Use of unprotected and enteric-coated enzymes in combination

Another approach to improve the results of enzyme therapy is to take advantage of the benefits of both unprotected and enteric coated formulations. Unprotected enzymes mix well with the meal and initially provide high duodenal lipase activity and fat digestion. However, depending on the acid secretory ability of the patient, when the gastric pH falls below 4, lipase will be inactivated providing a pattern of "effective early-ineffective late" therapy[32,33,51]. This pattern can be overcome by inhibiting acid secretion and using antacids to raise the pH to extend the duration of high pH gastric contents.

The pattern of effectiveness of enteric coated beads is one of "ineffective early - effective late". Combining the two approaches by starting therapy with unprotected enzymes followed by coated formulations would theoretically achieve a pattern of "effective early and effective late" and provide enzymes in parallel with gastric emptying of nutrients. We previously recommended this approach based on our experience[94]. The concept is supported and was given a firm physiologic basis by the exquisite studies by Gow *et al*[32] and Delchier *et al*[33] who used gastric and duodenal intubation to evaluate duodenal pH, enzyme and bile acid concentrations, and intraluminal digestion combined with fat balance studies. Meyer *et al*[37] also recommended the combination of unprotected and coated enzymes based on their elegant studies of emptying of enteric coated microbeads. To our knowledge no one has taken up the challenge of further investigating the combination approach, possibly because the recent focus has been on obtaining regulatory approval for new products rather than optimizing their effectiveness. More efficient use of available products would also require less enzyme and thus lower sales. The recent availability of an approved uncoated product (Viokaze) now makes testing the hypothesis possible.

Putting it all together

Based on perfusion studies and on theoretical grounds it has been suggested that 25000 to 50000 USP units of lipase should be administered per meal to achieve normal fat digestion and absorption[22]. As shown above, experience with pancreatic enzyme therapy with individual patients has shown that 18000 to 30000 USP lipase units per meal is probably the minimum needed for complete resolution of steatorrhea. Clinical trials with patients always trump laboratory experiments, and theoretical models and trials are needed to test and confirm hypotheses regarding most efficient use of enzymes. The one common feature of studies that has shown complete correction of steatorrhea is the presence of active lipase in the intestines for long periods, either because of the administration of unprotected enzymes or dissolution of enteric coated products in the stomach and their continued activity because the pH remained high[13,28,33]. The enteric coated product studied by Delchier *et al*[33] (Eurobiol 25000®) was very slow to dissolve after it reached the small intestine such that the amount of lipase measurable at the ligament of Treitz was similar to that following placebo. In contrast, those with high intragastric pH and rapid gastric emptying had high levels of intraduodenal lipase as well as intraduodenal absorption of triglycerides. Because a significant proportion of fat is emptied during the first 30 min of the meal, it is critical to provide exogenous lipase during that period. Potential approaches to solving this problem include: (1) the use of antacids and antisecretory drugs to prevent intragastric acidification; (2) administration of uncoated enzymes and possibly

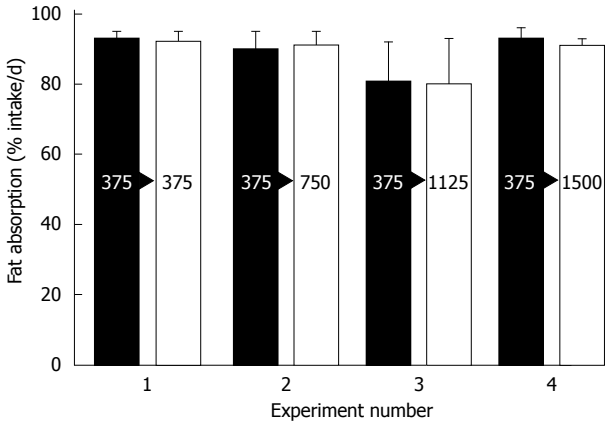

Figure 16 Data from 4 studies in children with cystic fibrosis comparing 375 USP lipase units/kg/meal to higher doses for the effect on steatorrhea. The results did not show a consistent effect on increasing the lipase dosage of an enteric coated preparation (Pancreaze®). Mean plus standard deviation are shown[79].

some sodium bicarbonate at the start of the meal; or (3) identify a strategy of emptying enteric coated products in the earliest portion of gastric emptying (for example, administer them before and during the meal). The dissolution characteristics of enteric coated products need further evaluation to examine when, where, how rapidly, and how completely the enzymes are released, and how these data relate to their clinical effectiveness.

Similarly, further studies are needed to address which changes in the timing of administration of pancreatic enzymes best coordinate pancreatic enzymes with emptying of gastric contents. For example, in three recent reviews the recommendations vary from 50% at the beginning of the meal and 50% at mid-meal[95], to during or immediately following the meal[96] and 25% with the first bite, 50% during the meal and 25% with the last bite[97]. From the available data and the data showing that a considerable amount of fat is emptied in the first hour, it is prudent when using enteric coated microbeads to start therapy just before the meal so that some microbeads are emptied during the first hour, then distribute the remaining enzymes throughout the meal. Those with hyperacidity may also benefit from adjuvant antisecretory therapy to reduce the duodenal acid load. However, it may not be possible to find an ideal schedule if one is restricted to using only enteric coated microbead therapy. Below we will discuss the available experience with currently approved therapies.

It has been known since the earliest days of pancreatic enzyme therapy that the patients who reliably experience good response are those with limited or no acid secretion. While the research focus has long been on duodenal lipase levels[22] one must now also consider how much and whether intragastric lipolysis due to the exogenous lipase contributes to the outcome. It should be clear that we have moved beyond the current "better than placebo" era of research aimed at obtaining regulatory approval for commercial products, and now need to focus on understanding how to reliably provide therapy and how to best use the available products.

More is not better using modern formulations

As a general rule for both unprotected and enteric-coated beads, the effect on steatorrhea is not directly related to the amount of lipase administered (namely, that after a threshold response, any further increase in the amount of enzyme given provides little or no additional benefit). This phenomenon has resulted in misinterpretation of many studies. For example, consider an experiment where the same dose of lipase is given using two different formulations (*e.g.*, 10 capsules are compared to 1 of another) with both formulations providing the same quantity of lipase. If both produce the same reduction in steatorrhea, the investigators would be tempted to conclude that one could use the formulations interchangeably, provided that the same quantity of lipase was administered. However, if they had included controls with one-half and with double the quantity of enzyme, they would likely have achieved the same result. This trap was revealed by studies examining whether there was a lipase dose - fecal fat responses (*e.g.*, Figures 12-16)[4,5,8,65,79,81,82]. For example, administration of 8000, 20000 or 32000 units of lipase using three different preparations of an enteric-coated commercial product produced no consistent change in fat malabsorption[65] (Figure 14). Figures 12, 13, 15, and 16 show more recent examples with a variety of enteric-coated products[4,5,8,81,82,98]. Figure 16 is especially revealing: in this study 4 subjects per group (children with cystic fibrosis) received therapy with 375 units of lipase/kg per day and then were given a different dose of 375, 750, 1125, or 1500 units/kg per day[79]. Clearly, the results with increasing to higher doses were almost identical.

Marketing strategies of companies selling pancreatic enzymes include attempts to link the amount of lipase required to fat intake and suggest that providers or patients increase the dosage in response to an unsatisfactory clinical response. Except for the low dosage products (which are priced about twice as high), enteric-coated pancreatic enzymes are currently priced between $2 and $4 per 10000 lipase units (Table 3). The lack of studies showing "more is better" and lack of head-to-head comparisons makes choice of therapy a matter of judgment.

Adding microspheres to food or putting them down feeding tubes

Enteric coated products to be taken orally are designed to dissociate when the pH is 5.5 or greater. The Cystic Fibrosis Foundation recommendations are consistent with the current package inserts: for infants and patients that are unable to swallow, recommended administration is to open the capsules and sprinkle its contents onto soft food mixtures with pH of 4.5 or less (*e.g.*, applesauce). The recommendation is based on theory rather than analysis of interaction of the enteric coating with complex formulations such as food. Sackman *et al*[99]. addressed the issue of mixing enteric-coated pancreatic enzymes with various food contents at various pH. They incubated en-

Table 5 Data needed to understand how to use new enzyme formulations

Results of all studies should not be withheld but should be published and/or placed on Clintrials.gov within 1 yr of completion
Trial data should provide the primary efficacy endpoint (*e.g.*, coefficient of fat absorption) as mean, standard deviation, median, range, and proportion with coefficient of fat absorption > 90% as well as proportion with coefficient of fat absorption < 85%
Gastric emptying of enteric coated pellets studied for all products are needed and the data should be published and/or placed on Clintrials.gov within 1 yr of completion
Kinetics of dissolution of enteric-coated microbeads in intestinal fluid or simulated intestinal fluid are needed and should include data pH's starting at approximately pH 5 through 7 at increments (*e.g.*, approximately 0.2 pH units)

Table 6 Recommended clinical trials

Head to head comparisons of different formulations within a product line as well as between commercial products
Comparative trials using different patterns of administration in relation to meals of enteric coated products (*e.g.*, before and during)
Studies combining unprotected and enteric coated preparations
Studies of unprotected preparations combined with maintenance of the intragastric pH constantly above 4
Initial pilot studies using ^{13}C-mixed triglyceride breath testing to test proof of concept may be the most efficient means of identifying which studies to test in human clinical trials

teric coated enzymes in saline, various food products with pH ranging from 5.6 to 6.5, and applesauce with pH of 3.4 and measured dissolution time as a surrogate for the integrity of the enteric-coating. Trypsin activity was used as a surrogate for lipase release. Among the foods tested, only applesauce reduced the integrity of the enteric-coating[99]. That study was conducted in 1982 with an older formulation but showed that theory is always subject to confirmation by experimentation. Studies with newer formulations are needed. Until that time it is likely that mixing with any food would be safe, although applesauce should probably be avoided. Shlieout *et al*[100] in an *in vitro* study mixed Creon 12000® in various baby foods with pH 4.5 or less to study use of pancreatic enzyme activity after passing it through various G-tubes. They found that the 16F Kimberly-Clark MIC-KEY tube was the smallest diameter tube that allowed passage of all food mixtures without clogging. Using tubes from other manufactures, they found that only 18F and larger tubes were able to pass all food content without clogging. All preparations retained 89.9% to 96.9% of the expected lipase activity. Nicolo *et al*[101] published 4 cases of patients dependent on enteric feeding and pancreatic enzyme supplementations. They reported that mixing pancreatic enzyme in all vehicles, including saline, applesauce, and fruit juices resulted in clogging of the tube; however, mixing the pancreatic enzyme in 8.4% solution of bicarbonate was effective. Interestingly, the combined use of pancreatic enzymes and bicarbonate is a common method used to unclog feeding tubes[102].

Recommended therapy

For the average patient, we recommend three, approximately 10000 USP units of lipase containing enteric coated microbead capsules/tablets per meal and one with snacks (*e.g.*, approximately 40000 USP units for an adult). The first dose is given before meals and the others during the meal. Following an unsatisfactory response one might consider adding approximately 20000 units lipase during meals. There are no data that increasing the dosage further increases effectiveness and is likely "beating a dead horse". Instead one should consider changing to a product with different characteristics (*e.g.*, from a microsphere to a minitablet), adding a unprotected enzyme product at the start of the meal, and/or adjuvant therapy with an PPI and/or sodium bicarbonate. As noted previously, one-third to more than one-half of patients will require therapy to be individualized. One should also consider the possibility of a second cause of malabsorption such as celiac disease or bacterial overgrowth. Treatment success should be assessed clinically and whenever available by an estimate of fat absorption. Longer term success should also be monitored in terms of maintenance of normal levels of fat soluble vitamins.

CONCLUSION

Hopefully, the current era of studies primarily targeted to obtaining FDA approval and marketing new products will soon transition into an era focusing on overcoming the remaining barriers that have limited the overall effectiveness of pancreatic enzyme therapy. In many ways we have not progressed beyond what was known in the 1980's. There are many options that potentially would improve current therapy and we have outlined a number of possibilities (Tables 5 and 6). A number of options need further testing, including the effects of combining unprotected enzymes (given with the first few bites and/or with sodium bicarbonate to buffer residual acid) in combination with enteric coated enzymes given throughout the meal. Hopefully comparative studies and studies of gastric emptying and dissolution of each formulation during normal meals will be done, and that results of those studies will be published in a timely manner.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Small bowel adenocarcinoma and Crohn's disease: Any further ahead than 50 years ago?

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Abstract

This review of the literature on small bowel carcinoma associated with Crohn's disease specifically addresses the incidence, risk factors, and protective factors which have been identified. It also reviews the clinical presentation, the current modalities of diagnosis, the pathology, treatment, and surveillance. Finally, the prognosis and future direction are addressed. Our experience with small bowel adenocarcinoma in Crohn's disease is reported. Readers will be provided with a better understanding of this rare and often poorly recognized complication of Crohn's disease.

Key words: Crohn's disease; Small bowel adenocarcinoma; Cancer risk; Cancer malignancy; Incidental carcinoma; Late complications of Crohn's disease; Inflammatory bowel disease

Core tip: This review of the literature on small bowel carcinoma associated with Crohn's disease specifically addresses the incidence, risk factors, and protective factors which have been identified. It also reviews the clinical presentation, the current modalities of diagnosis, the pathology, treatment, and surveillance. Finally, the prognosis and future direction are addressed. Our experience with small bowel adenocarcinoma in Crohn's disease is reported. Readers will be provided with a better understanding of this rare and often poorly recognized complication of Crohn's disease.

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INTRODUCTION

Over the past several decades it has become increasingly recognized that small bowel adenocarcinoma is an undeniable complication of Crohn's disease of the small intestine. The exact magnitude of this risk is virtually impossible to determine. The first case of small bowel carcinoma in Crohn's disease was reported by Ginzburg in 1956. Since then, there have been countless published case reports as well as numerous retrospective reviews and cohort studies which have attempted to define the occurrence of small bowel carcinoma in Crohn's disease.

This review of the literature on small bowel carcinoma associated with Crohn's disease specifically addresses the incidence, risk factors, and protective factors which have been identified. It will also review the clinical pre-

sentation, the current modalities of diagnosis, the pathology, treatment, and surveillance. Finally, the prognosis and future direction will be addressed. Our experience with small bowel adenocarcinoma in Crohn's disease is reported. Readers will be provided with a better understanding of this rare and often poorly recognized complication of Crohn's disease.

INCIDENCE

The relative risk of developing carcinoma of the small bowel in patients with Crohn's disease has been estimated to range from 6 to 320[1]. Jess *et al*[2] studied a population-based cohort of 374 patients with Crohn's disease in order to determine the long term risk of intestinal and extra-intestinal malignancies. The risk of small bowel adenocarcinoma was increased by more than 60 fold as compared to the general population. On the basis of 5000 cases of Crohn's disease reported in the world literature, Amman[3] calculated an association with carcinoma of 0.08%, which, he emphasized, was lower than the incidence of carcinoma of the small bowel in the absence of Crohn's disease, which he estimated at 0.098% in 137124 autopsies. However, the incidence in Crohn's disease may be higher than reported as the associated carcinoma may have been missed due to inadequate histopathologic study. These observational series serve to underline the possible fallacies of mass statistics collected from the literature.

Our review of the literature revealed 220 reported cases of adenocarcinoma associated with small bowel Crohn's disease. Since we do not have the number of patients who suffered from Crohn's disease of the small bowel from which those patients were extracted, the actual incidence of carcinoma associated with Crohn's cannot be calculated. These numbers were obtained through a Medline search from the years 1975 to 2013. Some of the reports were reviews and hence there may be some duplication of cases in the series reported.

There is nevertheless a general consensus that the risk of developing small bowel adenocarcinoma is greater in patients with Crohn's disease than in the general population. The exact magnitude of the increased risk is difficult to determine because information derived from population-based studies, case-controlled studies, physician surveys, and case reports have been combined.

Many reports have documented that adenocarcinoma of the small bowel is a complication of Crohn's disease and this has been well reviewed by Kerber[4] and Frank[5]. Sometimes the patients who develop an adenocarcinoma are those with small bowel-limited Crohn's disease but most often there is a combination of those with both small and large bowel Crohn's disease. Patients with longstanding small bowel Crohn's disease are thought to have an increased risk of small bowel carcinoma[6-9].

In a meta-analysis by von Roon *et al*[10], the relative risk of developing small bowel carcinoma in the 9642 patients was 28.37. The incidence rate of small bowel carcinoma in 12740 patients was 1.55 per 100000 patient years. In the review by Von Roon, the mean duration of Crohn's disease before the onset of carcinoma was 9 years (range 0.8-41). The relative risk of developing small bowel carcinoma compared to the background population was higher in North America (RR = 41.23), the United Kingdom (RR = 40) and Scandinavia (RR = 21.3).

Shaukat *et al*[11] studied all cases of small bowel carcinoma in persons 67 years and older in the Surveillance Epidemiology and End Results catchment area. They identified 923 cases of small bowel carcinoma and 142273 controls and found a strong association between Crohn's disease and small bowel carcinoma [odds ratio (OR) = 12.02]. The prevalence of Crohn's disease in patients with small bowel carcinoma was low (1.6%) so the absolute risk remains low.

RISK AND PROTECTIVE FACTORS

Numerous risk factors for developing small bowel carcinoma in Crohn's disease have been postulated in the literature. Many purported risk factors surfaced as a result of observed trends across case reports such as previous strictureplasty[12-15] and excluded/bypassed bowel segments[16-18]. Approximately 30% of reported small bowel carcinomas in Crohn's disease occurred in patients who had bypassed loops[19]. This complication stresses the need to encourage resection rather than bypass. Lashner[20] reported a case control study of carcinoma of the small bowel in Crohn's disease in which each case was matched to 4 randomly selected controls from an inflammatory bowel disease registry. The following factors were significantly associated with small bowel carcinoma in Crohn's disease: (1) occupation, with three cases having had exposure to halogenated aromatic compounds and aliphatic amines, asbestos, and cutting oil solvents and abrasives; and (2) 6-mercaptopurine use (OR = 10.8).

Several studies reported that the risk of developing small bowel carcinoma was according to the anatomic location of the Crohn's disease (Table 1). For example, the odds ratio of developing small bowel carcinoma was found to be much higher in 363 patients whose disease was confined to the small bowel (RR = 158.5) than in the 507 patients with ileocolic Crohn's disease (RR = 83.8)[10].

Table 1 displays a review of the studied risk factors for small bowel carcinoma in Crohn's disease in the literature, highlighting which authors agree and disagree with a purported risk factor. Clinicians should perhaps be more vigilant for small bowel carcinoma in patients with inflammation restricted to the small bowel versus ileocolic inflammation.

Protective factors against development of small bowel carcinoma in Crohn's disease have been less frequently studied, but available information is cited in Table 2. In a study of 29 patients with Crohn's disease and small bowel carcinoma Piton *et al*[21] found that small bowel resection and prolonged use of salicylates may protect against small bowel carcinoma in Crohn's disease patients.

Table 1 Risk factors for small bowel cancer in Crohn's disease

		Risk factors for small bowel cancer?	
		Yes	No
Crohn's related risk factors			
1	"Long" duration of CD	Greenstein *et al*[68] (mean 33.5 yrs) Greenstein *et al*[24] (mean 22 yrs) Jess *et al*[2] (median 16 yrs) Kamiya *et al*[69] (one case 18 yrs) Kersting *et al*[65] (one case 16 yrs) Kvist *et al*[70] (mean 29 yrs) Mellemkjaer *et al*[71] (mean 22 yrs) Michelassi *et al*[55] (mean 19.6 yrs) Mizushima *et al*[72] (mean 14 yrs) Munkholm *et al*[8] (mean 13.5 yrs) Palascak-Juif *et al*[27] (median 15 yrs) Petras *et al*[46] (mean 20 yrs) Ribeiro *et al*[1] (mean 26.5 yrs) Savoca *et al*[62] (one case 20 yrs) Sigel *et al*[48] (median 12 yrs) Solem *et al*[26] (median 21 yrs) Widmar *et al*[23] (mean 25.3 yrs)	Jess *et al*[73] Laukoetter *et al*[63]
2	Area of CD inflammation	Jess *et al*[2] Michelassi *et al*[55] Mizushima *et al*[72] Munkholm *et al*[8] Palascak-Juif *et al*[27] Ribeiro *et al*[1] Savoca *et al*[62] Solem *et al*[26] von Roon *et al*[10]	Laukoetter *et al*[63]
3	Jejunal CD	Lashner[20]	Palascak-Juif *et al*[27] Solem *et al*[26]
4	Strictures	Jaskowiak *et al*[15] Kersting *et al*[65] Lakatos *et al*[74] Petras *et al*[46] Ribeiro *et al*[1]	Solem *et al*[26]
5	Fistula	Kersting *et al*[65] Laukoetter *et al*[63] Ribeiro *et al*[1]	Lashner *et al*[20] Solem *et al*[26]
6	Bypassed segment	Greenstein *et al*[75]	Lashner[20] Palascak-Juif *et al*[27] Ribeiro *et al*[1] Solem *et al*[26]
7	CD Medications	[1]Lashner[20]	Canavan *et al*[76] Solem *et al*[26]
8	"Young" age	Freeman *et al*[60] (mean 45.7 yrs) Hoffman *et al*[7] (mean 46 yrs) Kersting *et al*[65] (one case 34 yrs) Laukoetter *et al*[63] (20 yrs earlier) Michelassi *et al*[55] (mean 47.7 yrs) Palascak-Juif *et al*[27] (median 47 yrs) Petras *et al*[46] (mean 46 yrs) Savoca *et al*[62] (mean 38 yrs) Sigel *et al*[48] (median 42 yrs) Widmar *et al*[23] (mean 55.4 yrs)	Jess *et al*[73] (median 66 yrs) Munkholm *et al*[8] (mean 70.5 yrs)
General risk factors			
9	Gender		Jess *et al*[2] Petras *et al*[46]
10	Male	Lakatos *et al*[74] Michelassi *et al*[55] Shaukat *et al*[11] Sigel *et al*[48] Ribeiro *et al*[1] Widmar *et al*[23]	Lashner[20] Palascak-Juif *et al*[27]
11	Female	Freeman *et al*[60]	
12	Black race	Shaukat *et al*[11]	
13	Past corticosteroid use		Kaerlev *et al*[67]
14	Past use of radioactive medication		Kaerlev *et al*[67]
15	Liver disease (cirrhosis/ hepatitis)		Kaerlev *et al*[67]
16	Gallstones		Kaerlev *et al*[67]
17	Previous cholecystectomy	Chen *et al*[64]	Kaerlev *et al*[67]
18	Prior history of peptic ulcer disease	Chen *et al*[64]	Kaerlev *et al*[67]
19	Celiac disease	Kaerlev *et al*[67]	
20	Prior malignancy		Solem *et al*[26]
21	Blood type B		Chen *et al*[64]
22	Rh type		Chen *et al*[64]
23	Tobacco	Chen *et al*[64] Lakatos *et al*[74]	Chow *et al*[77] Negri *et al*[25]
24	Alcohol	Chen *et al*[64]	Chow *et al*[77] Negri *et al*[25]
25	Diet	[2]Negri *et al*[25] [3]Chow *et al*[77]	
26	Lower education level	Kaerlev *et al*[67]	
27	Geographic location	[4]von Roon *et al*[10]	
28	Hazardous occupation	[5]Lashner[20]	
29	Marital status		Chen *et al*[64]
30	Religion		Chen *et al*[64]
31	Room type		Chen *et al*[64]

[1]6-MP; [2]Bread, pasta, rice, sugar, red meat; [3]Red meat, salt-cured foods, smoked foods; [4]North America higher risk; [5]Exposure to halogenated aromatic compounds and aliphatic amines, asbestos, solvents, oils, abrasives. CD: Crohn's disease.

CLINICAL PRESENTATION

Obstruction is the most common presenting manifestation in small bowel carcinoma in Crohn's disease with symptoms of nausea, vomiting and abdominal pain. Other possible presentations are hemorrhage, fistula, or perforation[22-24]. Unfortunately, all of these symptoms are hard to differentiate from those of a Crohn's exacerbation, which partly explains the challenge of detecting small bowel carcinoma in this patient population and results in the majority of diagnoses being made at the time of operation or postoperatively. In fact, only a small minority (< 5%) is diagnosed preoperatively[22]. Furthermore, Collier *et al*[19] described that over 50% of small bowel carcinomas in resected Crohn's disease segments were unsuspected or incidentally found by the pathologist.

Table 2 Protective factors for small bowel cancer in Crohn's disease

		Protective factor against small bowel cancer?	
		Yes	No
1	Diet	[1]Negri *et al*[25]	[2]Chow *et al*[77]
2	5-ASA	Piton *et al*[21] Solem *et al*[26]	
3	CD medications		Canavan *et al*[76] Solem *et al*[26] (other than 5-ASA)

[1]Coffee, fish, vegetables, fruit; [2]Fruits and vegetables. 5-ASA: 5-aminosalicylic acid.

Two important clinical indicators of malignancy are recrudescent symptoms after long periods of relative quiescence and small bowel obstruction that is refractory to medical therapy[23]. Therefore, it is prudent to consider a surgical assessment of patients with longstanding symptomatic Crohn's disease who fail to respond to conservative management.

The usual age of diagnosis of small bowel carcinoma in Crohn's disease patients is 45 to 55 years[4,6,7,22,23]. This is in contrast to small bowel carcinoma *de novo* which is usually diagnosed between 60 and 69 years of age[25]. Crohn's disease will often predate the carcinoma diagnosis by 20 to 25 years[4,22-24,26].

Palascak-Juif *et al*[27] studied 20 patients with Crohn's disease-associated small bowel carcinoma recruited from French university hospitals and compared them to 40 patients with small bowel carcinoma *de novo* recruited from a population-based registry. Small bowel carcinoma occurred after a median time of 15 years of Crohn's disease and was located within the inflamed areas of the ileum (19) or jejunum (1), whereas in patients with small bowel carcinoma *de novo* it was distributed all along the small intestine. The median age of diagnosis of small bowel carcinoma was 47 years (range 33-72 years) in patients with Crohn's disease and 68 years (range 41-95 years) in those with small bowel carcinoma *de novo*. The cumulative risk of small bowel carcinoma was 0.2% and 2.2% after 10 and 25 years of small bowel Crohn's disease, respectively. The diagnosis was made preoperatively in 1 of 20 patients with Crohn's disease and 22 of 40 patients with small bowel carcinoma *de novo*. Signet ring cells were found in 35% of Crohn's disease cancers but not in patients with small bowel carcinoma *de novo*. Relative survival at 2 and 5 years was not significantly different between these two categories of patients (54% *vs* 37% and 35% *vs* 30%; with and without Crohn's disease, respectively).

In a retrospective review from 1993 to 2009, Widmar *et al*[23] identified 29 patients with small bowel carcinoma (22 ileal and 5 jejunal) in Crohn's disease. There were no carcinomas in excluded intestinal loops. The median age of onset of Crohn's disease symptoms was 25 years and the median age at cancer diagnosis was 55.4 years, for a mean interval of 25.3 years. Widmar found that 75% of carcinomas arose in the terminal ileum, a location that only accounts for 13% of sporadic small bowel adenocarcinomas[28]. Patients with Crohn's disease developed adenocarcinoma at an average age of 48 years versus 65 in the general population, with a male to female ratio of 3 to 1.

Solem *et al*[26] described the clinical features, outcomes, and risk factors of small bowel carcinoma in Crohn's disease. Nine cases (4 males) were identified. The patients presented with abdominal pain (89%), obstruction (89%), and weight loss (78%). The carcinoma was located in the ileum in 8 patients (89%) and in the jejunum in 1 patient (11%). All cases but one had advanced disease with either lymph node involvement or metastases. The mortality rates at 1 and 2 years were 42% and 61%, respectively.

Floch *et al*[29] reviewed 47 previously reported small bowel carcinomas in Crohn's disease. The average age of the Crohn's carcinomas was 46.5 years while that for the *de novo* group was 55 years. The sexual ratios were 2.46:1 and 2:1 males to females for the respective groups. The *de novo* carcinomas had a slight predilection for the duodenum (4.7%) while the latter group had a heavy predilection for the ileum (70.8%) and contained no duodenal carcinomas. The prognosis of the Crohn's group appeared to be much worse than that of the *de novo* group with five year survivals of 3.7 and 20%-22%, respectively. Late diagnosis in the enteritis group was felt to be the major reason for this.

Hoffman *et al*[7] also reviewed the literature and found 49 cases and added two of their own. The Crohn's associated carcinomas differed from carcinoma not associated with Crohn's in that (1) mean age of carcinoma discovery was less (46 years *vs* 64 years); (2) more carcinomas arose in the ileum (76% *vs* 27%); (3) diagnosis and cure were less successful; and (4) they occurred more frequently.

The review by Fresko *et al*[30] of 59 reported cases of carcinoma of the small bowel in Crohn's disease, to which they added three of their own cases, revealed that (1) carcinoma develops at a younger age than in carcinoma *de novo*; (2) there is no difference in incidence of carcinoma in the first, second and third decades after onset of symptoms of Crohn's disease; (3) 73% of neoplasms arose in the ileum; (4) in all but one case it developed in inflamed segments of bowel; and (5) in 31% of cases carcinoma developed in a bypassed segment of bowel. They concluded that Crohn's carcinoma is a complication of Crohn's disease and not a chance co-existence of the two diseases in the patient.

DIAGNOSIS

The clinical diagnosis of small bowel carcinoma in Crohn's disease patients based on symptoms and physical examination is quite difficult, if not impossible. Indeed, many patients with carcinoma of the small bowel are not suspected of having a malignancy even at time of operation[5,6,31-33]. Most cases of small bowel carcinoma have been in segments involved with Crohn's disease. These malignancies were indistinguishable radiologically from

longstanding Crohn's disease. In two of the patients described by Kerber[4] and Frank[5], there was "shouldering", destruction, and a mass.

In general, imaging techniques may miss small lesions and may not be able to differentiate areas of small bowel carcinomas from those of severe Crohn's disease. Routine computed tomography (CT) exposes patients to radiation, and although magnetic resonance (MR) imaging does not, it is time consuming and costly[34]. Buckley *et al*[35] found CT staging of small bowel carcinoma to be 47% accurate but errors occurred in patients with Crohn's disease. Enteroclysis is invasive and requires special training[36]. The usefulness of FDG-PET is limited by the background chronic inflammation of Crohn's[37].

Video capsule endoscopy is challenged by issues of visualization (*i.e.*, limited field of vision, non-continuous image capture, lesions hidden in folds), inadequate preparation, and the stenosing nature of Crohn's disease[36] that may prohibit a capsule from passing. Furthermore, lesion localization can be difficult and it does not allow for tissue sampling[38].

Enteroscopic techniques do allow direct visualization and tissue sampling but are invasive, labor intensive, and may be limited by the length and tortuosity of the small bowel. Intraoperative endoscopy is now reserved for lesions which are not accessible by balloon enteroscopy[39].

Despite their disadvantages, there are published cases demonstrating the utility of some of the techniques listed above. Placé and colleagues[40] observed two different patterns using MR-enterography: the first was a long, circumferential, asymmetric, and heterogeneous thickening of the ileum with a visible nodule on free induction echo stimulated acquisition images, and the other was a mass of the terminal ileum showing restricted diffusion on diffusion weighted MR imaging. Soyer *et al*[41] evaluated 7 patients with small bowel carcinoma, and on CT enterography the carcinoma was visible in five patients. Four different patterns were individualized including small bowel mass (2 patients), long stenosis with heterogeneous submucosal layer (2 patients), short and severe stenosis with proximal small bowel dilatation (2 patients), and sacculated small bowel loop with irregular and asymmetric circumferential thickening (1 patient). Stratification, fat stranding, and comb signs were present in 2, 2, and 1 patient(s), respectively. Nevertheless, adenocarcinoma may be completely indistinguishable from benign fibrotic or acute inflammatory strictures.

A case reported by Kodaira *et al*[42] highlighted the unique successful combination of PET/CT and double balloon enteroscopy for the diagnosis of small bowel carcinoma in a Crohn's disease patient. Van Weyenberg *et al*[43] found both MR enterclysis and video capsule endoscopy useful but they believe that MR enterclysis is the better option. Ultimately, a combination of methods is likely the present day solution.

On the basis of their study of patients in whom adenocarcinoma of the small bowel developed as a complication of Crohn's disease, Kerber[4] and Frank[5] concluded: (1) the development of adenocarcinoma is more likely to be seen in patients with longstanding disease; (2) classical radiographic appearance of carcinoma may not be seen; (3) a progressive change in radiographic appearance over time with the development of masses, fistulas, strictures and obstruction should raise the suspicion of coexisting carcinoma; (4) malignancy should be considered when there is a longstanding quiescent disease activity followed by a recrudesces of symptoms with concurrent radiographic changes; and (5) fistulas may be associated with carcinoma in two ways: a mass produced by carcinoma or carcinoma arising in chronic fistulas from Crohn's disease. Contrary to prior recommendations, they suggest that obtaining radiographic examinations to document changing patterns of disease displays an important role in the management of patients with Crohn's disease and may lead to earlier detection of complicating carcinoma thus improving prognosis in such patients.

PATHOLOGY

In contrast to *de novo* small bowel carcinomas which are most often in the duodenum (55%)[28], 75% of Crohn's related small bowel carcinomas are ileal[22,28]. Miller *et al*[44] noted that all small bowel carcinomas associated with Crohn's disease were ileal in location. Watanabe *et al*[45] published a summary of small bowel carcinoma within Crohn's disease up until 1991 documenting only adenocarcinomas and signet ring cell carcinomas (Table 3). Petras *et al*[46] reported four patients with small intestinal carcinoma: three with poorly differentiated or signet ring cell type carcinomas and one with mucinous type. All four patients had high grade dysplasia in the mucosa immediately adjacent to the carcinoma, supporting the dysplasia-carcinoma sequence believed to occur in Crohn's disease as with ulcerative colitis[47,48]. A wider variety of malignancies have been reported and are noted in Table 3, including sarcomas, lymphomas, and carcinoids.

TREATMENT

The treatment of choice is wide resection of the small bowel segment harboring the carcinoma as well as resection of the corresponding mesentery and lymph nodes[22]. Pancreaticoduodenectomy for lesions of the second or third portion of the duodenum and right colectomy for carcinoma of the distal ileum would be required[49].

Evidence regarding the value of adjuvant chemotherapy for small bowel carcinoma is sparse and consists mostly of small retrospective reviews. Most available data is from experience in managing ampullary adenocarcinoma. Fishman *et al*[50] reported response rates upwards of 30% in the palliative setting: 33% with Gemcitabine, 50% with 5-FU or Capcitabine, and 42% with Platinum- or Irinotecan-based therapy.

PROGNOSIS

The prognosis of Crohn's associated small bowel carcinoma varies among reported studies but has been noted

Table 3 Histopathology of small bowel cancers in Crohn's disease

Histology	Watanabe *et al*[45]	Update since 1991
Adenocarcinoma	61 cases reported up until 1991 as quoted by Watanabe	Barwood *et al*[14] Chan *et al*[78] Chen *et al*[64] Christodoulou *et al*[79] Dossett *et al*[22] Feldstein *et al*[49] Fell *et al*[80] Fielding *et al*[9] Gillen *et al*[81] Gusakova *et al*[82] Jaskowiak *et al*[15] Jess *et al*[73] Kamiya *et al*[69] Katsanos *et al*[83] Kersting *et al*[65] Koga *et al*[84] Kronberger *et al*[85] Lindgren *et al*[86] Mellemjaker *et al*[71] Menon *et al*[12] Michelassi *et al*[55] Palascak-Juif *et al*[27] Partridge *et al*[13] Ribeiro *et al*[1] Richards *et al*[54] Rubio *et al*[87] Sammartino *et al*[88] Sigel *et al*[48] Solem *et al*[26]
Sarcoma		Gollop *et al*[89] (leiomyosarcoma) Jess *et al*[73] (leiomyosarcoma) Fielding *et al*[9] (reticulum-cell sarcoma)
Local lymphoma		Jess *et al*[73]
Carcinoid		Chen *et al*[64] Kvist *et al*[70] Mellemkjaer *et al*[71] Savoca *et al*[62]
Poorly differentiated		Petras *et al*[46] Savoca *et al*[62] Simpson *et al*[47]
Signet ring	8 cases reported up until 1991 as quoted by Watanabe	

to be poorer than the *de novo* small bowel carcinomas[24]. Most small bowel carcinomas in Crohn's disease present at a younger age and are more diffusely and distally located than *de novo* carcinomas, usually making them undiagnosable at a curable stage. Indeed, two-thirds of cases present with intestinal obstruction. Greenstein[51] reported two year disease survival for small bowel carcinoma in Crohn's disease as 9% compared with 15%-25% for *de novo* carcinomas. Mortality for carcinoma in excluded bowel has been reported to be as high as 100%[51]. One report of carcinomas developing in small bowel Crohn's strictures found only 9 such cases[52]. All patients had Crohn's disease for more than ten years. The average age of patients was 48 years compared to 65 years for *de novo* carcinomas. In patients with Crohn's disease, carcinoma affects the ileum twice as commonly as the jejunum and four times as commonly as the duodenum. Fifty-nine percent of all carcinomas complicating Crohn's disease were discovered incidentally during pathologic examination of resected specimens. If small bowel alone is considered this figure rises to 70%.

Small bowel carcinomas associated with Crohn's disease tends to be poorly differentiated and are associated with a poor prognosis[4,5,19,33,53]. Two year survival rates have been found to be as low as 27%[22]. In a report by Richards[54] with three ileal carcinomas, survival ranged from 8 to 44 mo.

Michelassi *et al*[55] reported 14 cases of intestinal carcinoma complicating Crohn's disease, 7 occurring in the small intestine and 7 in the large bowel. Two thirds of patients were male. The average age at time of diagnosis of Crohn's disease and carcinoma was 28 and 48 years respectively. In five patients with small bowel carcinoma the diagnosis was made at laparotomy. In the remaining cases only careful histologic examination revealed the carcinoma. Six small bowel carcinomas were located in the ileum. Two small bowel carcinomas were multi-focal and had surrounding mucosal dysplasia. No patient with regional or distal metastases survived five years in comparison with an 83% five year actuarial survival rate in patients with carcinoma confined to the intestinal wall. Mean survival was 6 mo for patients with small bowel carcinoma.

Hawker *et al*[53] reported the clinical and pathological details of three cases diagnosed between 1968-1980 with a review of 58 patients from the literature. Of the 61 cases, 41 carcinomas occurred in the ileum, 18 in the jejunum, 1 in the duodenum and ileum, and 1 in the ileum and colon. Eighteen occurred in bypassed intestinal loops. The prognosis was poor: 44 patients (72%) died with a mean interval of only 7.9 mo from the diagnosis of their malignancy.

Widmar *et al*[23] reported significant differences in the two year survival for node negative versus node positive carcinomas (79.3% *vs* 49%) and for localized versus metastatic disease (92.3% *vs* 33.3%). Overall, 36 mo survival was 69.3% compared to 40% among those without excluded loops. Sixteen patients had long periods of quiescent disease before the diagnosis (7-45 years) and 16 required operation for bowel obstruction that was refractory to medical management.

OUR EXPERIENCE

From 1990 to 2013, 10 patients with underlying Crohn's disease and small bowel adenocarcinoma were treated at our institution. In our series, there were twice as many males as females. The median age of Crohn's diagnosis was 28 years and the median age of small bowel adenocarcinoma diagnosis was 57 years; this interval is consistent with the literature. In none of the 10 patients was the diagnosis known preoperatively. Nine patients presented with a clinical picture of obstruction that did not respond to steroid treatment and required operation. Of the nine, two were found to have metastatic disease secondary to small bowel adenocarcinoma

at the time of operation, while the diagnosis for the remaining seven was made on final pathology examination. The tenth patient in our series had refractory Crohn's disease and was incidentally found to have terminal ileal adenocarcinoma on final pathology.

An equal number of patients had Crohn's disease isolated to their small bowel as concomitant small and large bowel disease. None of our patients had bypassed loops of small bowel. In fact, only three patients had had previous operations for Crohn's disease. All but one of our patients had terminal ileal adenocarcinomas; the exception was one jejunal carcinoma. Moreover, all patients had either stricturing and/or fistulizing Crohn's disease. Four of our 10 patients had been treated with 5-ASA, a reported protective factor. All patients had a history of remote immunomodulator use but none were on maintenance immunomodulators at the time of presentation; if they were receiving medical therapy, it was solely high dose steroids. This is consistent with the conclusions made by Kerber[4] and Frank[5] concerning the development of a small bowel adenocarcinoma after a period of quiescent Crohn's disease. As reported by others, the prognosis for small bowel adenocarcinoma in our series was also quite poor: the carcinoma-related mortality in our series is 70%.

SURVEILLANCE

It has become increasingly recognized that the risk of developing carcinoma of the colon in patients with colonic Crohn's disease is comparable to those with chronic ulcerative colitis. Hence, regular colonoscopic surveillance in search of dysplastic changes is in order. However, no similar surveillance for patients with small bowel Crohn's disease is possible.

Greenstein[24] suggests that surveillance should consist of regular abdominal examinations and that the recurrence of obstructive symptoms as well as the development of new symptoms should not be ignored especially after long quiescent periods.

CEA levels, found to be elevated in up to 38% of patients with active Crohn's disease[56,57], have not been found to be useful in monitoring for small bowel carcinoma[19,58].

LIMITATIONS

Each of the studies included in this descriptive view has its own limitations. A descriptive review such as ours could not possibly be exhaustive if it had strict inclusion/exclusion criteria. Thus we chose to include data and observations from all available studies despite their limitations.

It is difficult to draw conclusions regarding the cumulative incidence of small bowel carcinoma in Crohn's disease from studies with such a low frequency of event as small bowel carcinoma. Compared to current data, incidence values from older studies may actually be overestimated, as 5-ASA formulations (possibly protective against small bowel carcinoma) were released in the late 70's and 80's, late in the observation period of most cohorts[59]. Furthermore, the majority of reports did not have small bowel carcinoma as a primary outcome. Analysis did not routinely address incidence and discussions were sometimes not exclusive to the small bowel (*e.g.*, "intestinal"[60], "upper digestive tract"[61], or discussing risk factors for small bowel and colorectal carcinomas together[62]). Studies rarely controlled for immunosuppressive agents, tobacco or alcohol[63], and those that did examine such exposures did not provide quantitative data[64]. Finally, multiple biases inherent of retrospective and single-centre studies exist in the available literature. For example, a high incidence of small bowel carcinoma in Crohn's disease may reflect a bias in tertiary hospitals[65] or a surveillance bias due to close monitoring of Crohn's disease patients[66], and reported risk factors may be a result of recall bias[67]. These limitations are inherent to the challenging problem of a rare disease that is difficult to diagnose.

CONCLUSION

In this review, we highlighted the available current evidence and the gaps of knowledge, technology, and clinical guidelines required for improving care of Crohn's disease patients at risk of this devastating problem. Although the association of carcinoma in Crohn's disease and the need to screen Crohn's disease of the colon is well established, carcinoma associated with Crohn's disease of the small bowel is difficult to diagnose and indeed is often not identified until operation for what is believed to be an exacerbation or non-response to medical therapy. Sadly, the diagnosis is often made after careful examination of the resection specimen by the pathologist. Over the decades there has been a lack of significant improvement in prognosis. There is a need to elucidate screening modalities to facilitate earlier diagnosis and treatment.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Ulcerative colitis: From inflammation to cancer. Do estrogen receptors have a role?

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Abstract

Ulcerative colitis (UC) is a condition at increased risk for colorectal carcinoma (CRC) development. Nowadays, screening and follow-up programs are routinely performed worldwide to promote the early detection of CRCs in subjects with well known risk factors (extent, duration and severity of the disorder). The diffusion of these procedures is presumably the main reason for the marked reduction of cancer incidence and mortality in the course of UC. In addition, chemoprevention has been widely investigated and developed in many medical fields, and aspirin has shown a preventive effect against CRC, while mesalazine has been strongly invoked as a potential chemopreventive agent in UC. However, available studies show some limitations due to the obvious ethical implications of drug withdrawal in UC in order to design a control group. The estrogen receptors (ER) alpha/beta balance seems to have a relevant influence on colorectal carcinogenesis and ER beta appears to parallel apoptosis, and hence an anticarcinogenic effect. Phytoestrogens are compounds acting as ER beta agonists and have shown a promising chemopreventive effect on sporadic as well as genetically inherited CRC. There is evidence suggesting a role for ERs in UC-related carcinogenesis. In this perspective, since these substances can be considered as dietary supplements and are completely free from side effects, phytoestrogens could be an interesting option for CRC prevention, even when the disease is a consequence of long-term chronic inflammation, as in the course of UC. Further studies of their effects are warranted in both the basic research and clinical fields.

Key words: Ulcerative colitis; Epithelial dysplasia; Colorectal cancer; Estrogen receptors; Chemoprevention; Phytoestrogens; Dietary supplementation; Inflammatory bowel disease

Core tip: The present work outlines the main data regarding a possible involvement of estrogen receptors in colorectal carcinogenesis, paying particular attention to cancer arising in the course of ulcerative colitis. A protective role for beta receptors has been suggested by many studies. The challenge for the future could be to devise chemopreventive strategies against colorectal carcinoma employing estrogen receptor beta agonists, such as phytoestrogens.

Principi M, Barone M, Pricci M, De Tullio N, Losurdo G, Ierardi E, Di Leo A. Ulcerative colitis: From inflammation to cancer. Do estrogen receptors have a role? *World J Gastroenterol* 2014; 20(33): 11496-11504 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i33/11496.htm DOI: http://dx.doi.

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ULCERATIVE COLITIS: FROM INFLAMMATION TO CANCER

Ulcerative colitis (UC) is associated with an increased risk of colorectal cancer (CRC), which has been related to the long-standing chronic inflammation[1]. However, the magnitude of the risk is difficult to estimate, as many factors may bias study results[2] (*i.e.*, patient selection, number of patients, completeness of case recruitment and ascertainment and duration of follow-up)[2,3].

Castaño-Milla *et al*[4] reported an overall incidence rate of CRC in UC of 1.67/1000 per year of disease (PYD) and incidence rates per decade were estimated at 1.01/1000, 3.75/1000 and 5.85/1000 PYD for the first, second and third decades, respectively. In a meta-analysis of prospective population-based studies, Jess *et al*[5] found that an average of 1.6% of patients with UC were diagnosed with CRC during the first 14 years of follow-up, and the estimated standardized incidence ratio (SIR) was 2.39 (2.1-2.7). Recent time-trend studies also demonstrate a decreasing risk of CRC in UC patients[6]. In a recent meta-analysis[4] the incidence rate was found to have decreased from 4.29/1000 PYD in studies published in the 1950s to 1.09/1000 PYD in the studies published between 2000 and 2011.

As known, reported risk factors for CRC include extensive disease[7,8], young age at diagnosis[9], a family history of CRC[10], co-existing primary sclerosing cholangitis (PSC)[11] and persistent inflammation of the colon[12,13].

The pathophysiology of colitis-associated cancer suggests the action of numerous positive and negative regulators[14]. Positive regulators are pro-carcinogenic cytokines such as tumor necrosis factor alpha (TNF alpha), that is over-expressed in a murine model of carcinoma arising on colitis[15], interleukin (IL)-6[16] and IL-21[17] and chemokines such as CCL2, whose expression is enhanced by TNF alpha, causing the recruitment of macrophages and monocytes[18]. Negative regulators include IL-10[19,20], transforming growth factor beta (TGF beta)[21] and MyD88, a Toll-like receptor adaptor, that has been found to significantly reduce tumor number and size in the Apc$^{min/+}$ mouse model of intestinal tumorigenesis[22,23].

The progression from UC to CRC is a multistep process in which the accumulation of genetic mutations leads to the sequential evolution to low-grade dysplasia (LGD), high-grade dysplasia (HGD) and finally to cancer[24]. The p53 tumor suppressor gene appears to be a key factor in the initial steps of UC-associated colorectal carcinogenesis, being the most frequent single founding mutation in UC associated CRC[25]. p53 is overexpressed in 33%-67% of patients with dysplasia and in 83%-95% of patients with UC-associated CRC[26,27]. Other genes that undergo mutation in the following stages of carcinogenesis are kRAS, DCC, cyclin D, COX, iNOS, APC and beta-catenin (Figure 1), in a sequence that is substantially different from the classical adenoma-carcinoma pathway[28,29].

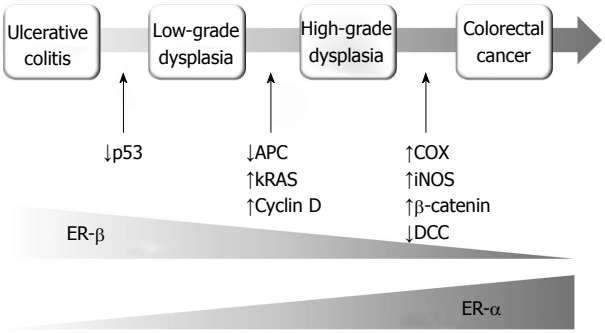

Figure 1 progressive steps of ulcerative colitis-related carcinogenesis, genetic pathways and estrogen receptors alpha and beta patterns.

The essential morphological features of dysplasia, are (1) nuclear alterations such as increased nuclear to cytoplasmic ratios and hyperchromasia; (2) depletion of goblet cells; and (3) abnormal architectural patterns corresponding to dysregulated cellular proliferation, such as glandular crowding, a villous architecture and diminished surface maturation. HGD differs from LGD in that there are additional alterations, *i.e.*, impaired cellular polarity including loss of nuclear parallelism, stratification of nuclei patterns such as a cribriform architecture. In most cases, the nuclei in HGD show severe cytological aberrations such as irregular nuclear membranes, abnormally prominent nucleoli or atypical mitotic figures[30]. The progression of such alterations is accompanied by both a progressive increase of epithelial proliferation and a reduction of apoptosis. This phenomenon starts as alterations of glandular architecture (*i.e.*, shortening, loss of parallelism, ramification and branching) which anticipate the dysplasia onset[31].

The potential risk of malignant degeneration of UC to CRC has made it necessary to institute surveillance protocols to achieve early recognition and treatment of dysplastic lesions. The current evidence-based consensus for endoscopy in inflammatory bowel disease[32] suggests that surveillance should start when the risk starts to increase, *i.e.*, after 8-10 years from the onset of disease[7]. This first colonoscopy also aims to reassess the extent of disease, since this parameter has an impact on the risk of CRC. After this first colonoscopy, patients with high risk features (stricture or dysplasia detected within the past 5 years, PSC, extensive colitis with severe active inflammation, or a family history of CRC in a first degree relative aged less than 50 years) should undergo surveillance colonoscopy annually. Conversely, patients with intermediate risk factors should have surveillance colonoscopy scheduled every 2 to 3 years and those without risk factors every 5 years. Biopsy sampling is fundamental: the American Gastroenterological Association recommends extensive sampling, of a minimum of 33 specimens[33], while, according to the British Society of Gastroenterology[34],two to four random biopsies every

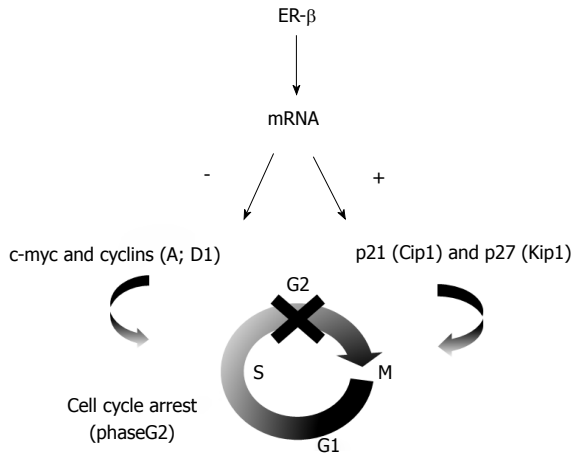

Figure 2 Estrogen receptors beta and interactions with genes involved in the regulation of cell cycle. Estrogen receptors (ER) beta has an antagonist inhibitory function, mediated by the down-regulation of proto-oncogenes c-myc and cyclins (as indicated by minus sign next to the arrow on the left side) and up-regulation of oncosoppressants p21 and p27 (as indicated by plus sign next to the arrow on the right side). In the lowest part of the figure is indicated the cell cycle phases and the site of its interaction with ER beta induced mediators (G2 phase).

10 centimetres should be taken.

Compliance to surveillance protocols, as well as a correct clinical overview of UC and the adequate pharmacological management of the disease, have led to a decreasing CRC incidence and mortality in UC[35,36]. In 1971, de Dombal[37] reported a 5% cumulative risk of CRC in a population from Leeds with extensive UC after 10 years and 41.8% after 25 years. Thirty years later, the cumulative risks reported by Lakatos *et al*[38] had dropped dramatically: 0.6% after 10 years, 5.4% after 20 years and 7.5% after 30 years of disease duration. These data testify to the exceptional impact of surveillance in the natural history of UC[39], but we must consider that it is not the only prevention strategy: other routes, such as chemoprevention, may have a remarkable effect.

ESTROGEN RECEPTORS

Modern medicine and oncology have been profoundly affected by the discovery of the estrogen receptors (ERs), a potential marker that plays a pivotal role in the pathogenesis, prognosis and therapy of various cancers, such as breast, prostate and colon. Estrogens can regulate the growth, differentiation, and function of various target tissues both within and outside the reproductive system[40,41]. The most relevant event after the initial discovery of these receptors[42] was the identification of two subtypes, ER alpha and ER beta, that are expressed at different levels in each organ of the human body[43]. Variations in the phenotype of knock-out mice lacking ER alpha or ER beta suggested that these receptors have different biological activities[44]. Moreover, *in vitro* and *in vivo* studies in ER beta knock-out mice demonstrated that ER beta is a modulator of ER activity, as it is able to reverse the effects of ER alpha and to inhibit estradiol-dependent proliferation[45,46]. These experiments demonstrated that ER alpha is a positive regulator of cellular growth, while ER beta has an antagonist inhibitory function, mediated by the down-regulation of proto-oncogenes (c-myc and cyclins) and up-regulation of oncosuppressants (p21 and p27), resulting in cell cycle arrest[47] (Figure 2). Experiments showing that in various cancers ER alpha is over-expressed and ER beta is down-regulated confirmed *in vitro* studies and demonstrated that cell proliferation is the result of a balance of ER alpha and ER beta[48,49].

ESTROGEN RECEPTORS AND COLORECTAL CANCER

The hypothesis of a possible link between CRC and ERs was advanced after the publication of epidemiological studies showing that females have a lower rate of colonic adenomas and cancers than males before menopause and that the differences progressively lessen after menopause[50]. Similarly, both observational and interventional data have shown that hormone replacement therapy decreases colonic adenoma and cancer risks[51,52]: in the last 40 years, a reduction of deaths from large bowel carcinoma has been observed in the United States. This reduction was significantly higher in women (30%) as compared to men (7%). In the same study, a link was observed between oral contraceptive use and a reduction of colorectal cancer, whereas there was a higher than expected frequency of colorectal tumors among non users[53].

After the demonstration by our group that ERs are expressed in the colonic mucosa[54], Konstantinopoulos *et al*[55] demonstrated that ER beta is highly expressed in normal colonic mucosa in humans, while it is significantly reduced in CRC; this reduction is more pronounced in the case of poorly differentiated tumors. Since the majority of CRCs are derived from adenomatous polyps (a precancerous condition) our group recently evaluated the expression of ER alpha and ER beta in the colonic tissue of 25 patients with adenomatous polyps of the colon and in 25 normal subjects[56]. ERs expression was then correlated to proliferation and apoptosis. Our data confirmed that ER beta is the prevalent estrogen receptor in normal mucosa and shows a significantly reduced expression in adenomatous polyps (Figure 3). In a successive study, we confirmed that ER beta plays a primary role in the regulation of colonic mucosa proliferation in patients affected by Familial Adenomatous Polyposis (FAP)[57], an inherited disease characterized by an early inclination to develop hundreds of polyps and consequently CRC. Furthermore, ERs can even influence the prognosis of CRC, as it has been demonstrated that patients affected by CRCs with a minimal ERs expression had poor prognosis and short survival[58].

All these data confirm that sex steroid hormones are involved in CRC development and suggest that ER beta could play an important role in the early phase of the carcinogenic process and hence could be a target in the primary prevention of CRC[59].

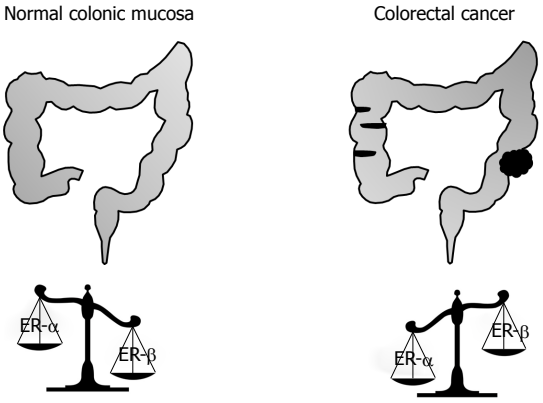

Figure 3 Alpha and beta estrogen receptors balance in normal and neoplastic colon.

ESTROGEN RECEPTORS EXPRESSION IN THE PROGRESSIVE STAGES OF ULCERATIVE COLITIS-RELATED CARCINOGENESIS

ER beta has been suggested to exert anti-inflammatory and anti-tumorigenic effects in the colon, providing a translational potential to prevent and/or treat inflammatory bowel disease (IBD) and its progression to colitis-associated CRC[60,61]. Most studies in this field used a consolidated animal model which accurately mimics the carcinogenic model related to chronic bowel inflammation in mice (*i.e.*, Azoxymethane/Dextran Sodium Sulfate - AOM/DSS)[62,63].

Saleiro *et al*[64] demonstrated that ER beta-deficient mice developed more severe clinical colitis compared to wild type mice, as evidenced by a significantly higher disease activity index after DSS treatment, as well as the inflammation score and grade of dysplasia. ER beta-deficient colons presented a greater number and size of polyps, and were characterized by a significant increase in IL-6, IL-17, TNF alpha and interferon-gamma mRNA levels as compared to wild type mice organs. Furthermore, higher protein expression levels of nuclear factor-kappa B, inducible nitric oxide synthase (iNOS), beta catenin, proliferating cell nuclear antigen, mucin-1, and significantly lower caveolin-1 and mucin-2 protein levels, were shown in ER beta knock-out mice compared to wild type. These data suggest a possible anti-inflammatory and anti-neoplastic mechanism of action of ER beta in UC-arisen CRC. These results suggest that ER beta may be protective in the AOM/DSS-induced CRC model in mice, supporting a preventive and/or therapeutic potential for the use of ER beta-selective agonists in IBD.

Fujii *et al*[65] performed a study to clarify whether methylation analysis of the ER gene in non-neoplastic epithelium can contribute to the prediction of an increased neoplasia risk in UC patients. The study was based on the assumption that the ER gene shows an age-related methylation in the colorectal epithelium and this phenomenon is frequently found in sporadic colorectal neoplasia, suggesting that it may predispose to colorectal neoplasia. The results suggested that the analysis of ER gene hypermethylation may be a potentially useful marker for identifying individuals at increased risk of neoplasia among those with long-standing and extensive UC. The same group confirmed that the quantitative analysis of ER gene methylation in non-neoplastic epithelium is a marker for identifying individuals at increased risk of neoplasia in long-standing and extensive UC[66].

A preliminary report by our group[67] assessed the pattern of ER-alpha/beta expression in relation to epithelial apoptosis and cell proliferation in long-lasting UC. We did not observe significant variations in ERs and their ratio in UC compared to UC-low degree dysplasia. However, there was a statistically significant progressive increase in apoptosis in UC and in UC-dysplasia that, despite Ki-67 expression, revealed a more marked significant increase at the same stages. This result, despite the small sample and the inclusion of only low-grade dysplasia, suggested that a possible ER-beta overseer of apoptosis/proliferation is operative until the investigated stage of carcinogenesis (Figure 1). In fact, in LGD we observed a high increase in cell proliferation with invariable levels of ER beta, accompanied by mild increased apoptosis, that was presumably unable to completely counter Ki-67 over-expression. Further, we investigated ER beta, ER alpha expression and their ratio in normal mucosa, in UC and in UC-low and high grade dysplasia and CRC. ERs did not show significant changes until LGD, while in HGD and UC-carcinoma there was a dramatic loss of ER beta expression and the ER beta/ER alpha ratio. Apoptosis and the TUNEL/Ki-67 ratio demonstrated a statistically significant progressive decrease from LGD to UC-carcinoma[68].

IS THERE A ROLE FOR CHEMOPREVENTION?

The main risk factors for colorectal cancer are not suitable targets for therapeutic intervention, but primary chemoprevention is an intriguing therapeutic option. The question whether mesalazine could exert a chemopreventive effect has been raised and various studies have investigated this aspect.

The mechanisms by which aspirin or non-steroidal anti-inflammatory drugs (NSAIDs) act in the chemoprevention of CRC in non-IBD patients have not been entirely elucidated. However, data on the chemopreventive effect of aspirin and NSAIDs and CRC are supported by a series of independent lines of evidence. Indeed, several epidemiological studies have shown an inverse correlation between aspirin intake and the risk of CRC[69-71]. Furthermore, studies on secondary chemoprevention reported that aspirin intake was associated with a decreased risk of adenoma recurrence[72,73]. Aspirin and NSAIDs seem to act by inducing apoptosis in the colonic epithelium through the inhibition of cyclooxygenase (COX) activity and arachidonic acid accumulation[74]. Recent evidence suggests that COX inhibition can also change the activity

of mitogen-activated protein kinases and NFκB[75,76].

The analogies between acetyl-salicylic acid and mesalazine (5-amino-salcylic acid), and the results obtained by using acetyl-salicylic acid as a chemopreventive agent in patients with sporadic colorectal cancer have prompted the study of potential chemopreventive effects of mesalazine in inflammatory bowel disease. The results of both epidemiological and experimental studies have shown that long-term 5-amino-salicylic acid treatments appear to have a chemopreventive effect. We can cite two studies, by Eaden and Lashner, in which the relative risk of CRC was estimated to be 0.18 and 0.88, respectively[77,78]. In a group of patients affected by UC and PSC, the risk was 0.88[79]. The evidence for this effect is provided by retrospective and case-control studies, however, whose results do not reach the highest grades for evidence-based recommendations. Indeed, not all clinical studies reported favorable results regarding CRC in IBD patients. Negative results were mainly reported in studies that elicited positive results with other drugs such as folate or ursodiol[80]. The peculiarities of the cohorts enrolled in these studies (disease refractory to conventional therapy, consideration for treatment with experimental therapy, consultation for surgery) may account for the negative outcome.

Positive results are supported by a series of experimental studies demonstrating the multiplicity of actions of 5-amino-salicylic acid, although data regarding the chemopreventive effect of 5-amino-salicylic acid may not be rigorous enough to meet the criteria for the highest evidence-based medicine recommendations. A final consideration is that suitable evidence may not be rationally gained in this case, because discontinuation of 5-amino-salicylic acid treatment would be unethical in patients with UC[81].

FUTURE PERSPECTIVES OF CHEMOPREVENTION BY BETA RECEPTOR AGONISTS

The data summarized in the previous sections suggest the hypothesis that the loss of ER beta expression could be a marker of colonic mucosa at increased risk for colonic neoplasia and that the induction of ER beta with ER beta-selective phytoestrogens could exert a chemopreventive effect against CRC.

Observational data also suggest that phytoestrogen intake may be associated with a decreased incidence of advanced lesions in both men and women[82-84]. The mechanism of the putative protective effect of estrogens and phytoestrogens on colonic neoplasia is not fully understood, but it seems to be markedly different from the one underlying the detrimental effect of estrogens in breast cancer. In the breast, it is well established that the detrimental effect is due to estrogen binding to the proproliferative ER alpha, since a similar effect is not found in women with ER-negative breast cancers[85].

Barone *et al*[86] have shown that the ER beta/ER alpha ratio was lower in the normal small intestinal mucosa of $APC^{min/+}$ mice than in syngenic APC wild type and this phenomenon was associated with a decreased apoptotic activity. The ER beta/ER alpha ratio and apoptosis were normalized by supplementation with a combination of silymarin and insoluble fibers. The combination also markedly decreased the number and size of intestinal tumors in $APC^{min/+}$ mice[86]. Silymarin displays a full ER beta agonist activity[87,88] and lignans also exert phytoestrogenic activity[89]. Another study by our group[90] was a randomized, double blind placebo-controlled trial in patients undergoing surveillance colonoscopy for previous sporadic colonic adenomas. Sixty eligible patients were randomized to receive a placebo or active dietary intervention with phytoestrogen supplements twice a day, for sixty days before surveillance colonoscopy. The phytoestrogen administration group showed a significant increase in ER beta protein and a general trend to an increase in ER beta, ER beta/ER alpha, TUNEL/Ki-67 ratio. Moreover, a significant increase of ER-beta protein, mRNA and labeling index (*i.e.*, the percentage of ER-beta positive cells at immunohistochemistry) and a decrease of ER-alpha protein, as well as an increase in ER beta/ER beta protein were observed in phytoestrogen versus placebo group in patients without recurrent polyps. Therefore, the role of ER beta on the control of apoptosis, as well as its amenability to dietary intervention, were supported by this study.

Finally, 90-d supplementation with phytoestrogens was efficacious in reducing polyp number and size in recurrent duodenal adenomas of patients with FAP with an ileal pouch-anal anastomosis[91].

CONCLUSION

UC is a condition that increases affected patients' risk for CRC development. Nowadays, specific screening and follow-up programs, based on epidemiological and clinical parameters, are routinely performed to promote the early detection of CRC onset. This practice has induced a marked reduction of the cancer incidence and mortality in subjects with UC.

Chemoprevention is an interesting topic which has been widely investigated and developed in many medical fields[92]. Aspirin has shown a preventive effect on CRC onset, and mesalazine has been strongly invoked as a potential chemopreventive agent against carcinoma arising in UC[93].

The ER alpha/beta balance seems to have a relevant influence on colorectal carcinogenesis and ER beta appears to parallel apoptosis, thus exerting an anti-carcinogenic effect[94]. In preliminary studies phytoestrogens, which are able to act as ER beta agonists, have shown promising chemopreventive effects on sporadic as well as genetically inherited CRC. In view of the strong evidence of a role for ERs in UC-related carcinogenesis, and taking into account the fact that phytoestrogens can

be considered as dietary supplements and are completely free from side effects, they offer interesting prospects for CRC prevention even when the disease is the long term consequence of chronic inflammation.

In conclusion, ERs have a role in the development of all different types of CRC[95] (sporadic, genetic and post-inflammatory). Their targeted use is, therefore, a fascinating field for both basic and clinical investigations in order to elucidate the underlying pathophysiological, prognostic and therapeutic aspects.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Intestinal microbiota, probiotics and prebiotics in inflammatory bowel disease

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Abstract

It has been presumed that aberrant immune response to intestinal microorganisms in genetically predisposed individuals may play a major role in the pathogenesis of the inflammatory bowel disease, and there is a good deal of evidence supporting this hypothesis. Commensal enteric bacteria probably play a central role in pathogenesis, providing continuous antigenic stimulation that causes chronic intestinal injury. A strong biologic rationale supports the use of probiotics and prebiotics for inflammatory bowel disease therapy. Many probiotic strains exhibit anti-inflammatory properties through their effects on different immune cells, pro-inflammatory cytokine secretion depression, and the induction of anti-inflammatory cytokines. There is very strong evidence supporting the use of multispecies probiotic VSL#3 for the prevention or recurrence of post-operative pouchitis in patients. For treatment of active ulcerative colitis, as well as for maintenance therapy, the clinical evidence of efficacy is strongest for VSL#3 and *Escherichia coli* Nissle 1917. Moreover, some prebiotics, such as germinated barley foodstuff, *Psyllium* or oligofructose-enriched inulin, might provide some benefit in patients with active ulcerative colitis or ulcerative colitis in remission. The results of clinical trials in the treatment of active Crohn's disease or the maintenance of its remission with probiotics and prebiotics are disappointing and do not support their use in this disease. The only exception is weak evidence of advantageous use of *Saccharomyces boulardii* concomitantly with medical therapy in maintenance treatment.

Key words: Gut; Microbiota; Inflammatory bowel disease; Probiotic; Prebiotic

Core tip: Intestinal microbiota seems to play an important role in the pathogenesis of inflammatory bowel disease. There is very strong evidence supporting the use of certain probiotics and prebiotics in the therapy of ulcerative colitis and pouchitis, whereas their beneficial role in Crohn's disease has not yet been proven. This article describes the role of gut microbiota in the pathogenesis of inflammatory bowel disease and delineates the possible mechanisms of certain probiotics and prebiotics in disease treatment and maintenance of remission.

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REVIEW OF THE FACTS ON GUT MICROBIOTA IN INFLAMMATORY BOWEL DISEASE

A variety of factors, which may be environmental, genetic, immunological, and microbial in nature, contribute to

the development of inflammatory bowel disease (IBD)[1]. Although the exact etiology of IBD remains unclear, it is believed to be the result of complex aberrant immune responses to as yet undetermined environmental factors (most likely intestinal microorganisms) in the gastrointestinal tract of genetically susceptible hosts[2].

The human gut normally hosts roughly 10^{14} bacterial organisms of up to 1000 different species; this bacterial community can add up to 1-2 kg[1]. In total, the number of intestinal bacteria is approximately ten times the number of cells constituting the human body, with the collective bacterial genome, also referred to as the microbiome, containing 100-fold more genes than the entire human genome[3,4]. More than 99% of the gut microbiota is composed of species within 4 bacterial divisions: *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria*[5,6]. Greater variations exist below the phylum level, and certain butyrate-producing bacteria, including *Faecalibacterium prausnitzii, Roseburia intestinalis*, and *Bacteroides uniformis*, have been identified as key members of adult gut microbiota[7]. The predominant species in the proximal small intestine are aerobic and Gram-positive. In the distal small bowel, Gram-negative species begin to outnumber Gram-positive bacteria[8]. Distally to the ileocecal valve, bacterial concentrations increase sharply[8], and the most densely populated region of the gastrointestinal tract is the colon, with up to 10^{12} bacteria per gram of intestinal content and a population consisting predominantly of the *Bacteroides, Bifidobacteria, Fusobacteria, Clostridia*, and *Peptostreptococci* groups[1]. The majority of intestinal bacteria belong to the phyla *Bacteroidetes* (64% of attached colonic species) or *Firmicutes* (23% of normal species)[1,5]. *Enterobacteriaceae* such as *Escherichia coli* are relatively minor components of the *Proteobacteria* division (8% of all bacteria)[5].

There is plenty of evidence supporting the hypothesis of the involvement of intestinal microbiota in IBD pathogenesis. Crohn's disease (CD) and ulcerative colitis (UC) tend to occur in the colon and distal ileum, which contain the highest intestinal bacterial concentrations[5]. A pathogenic role of luminal constituents is suggested by the prevention and treatment of Crohn's disease by the diversion of fecal stream and reactivation of inflammation within one week following reinfusion of ileostomy contents[9]. In patients with CD, division of the fecal stream proximally to the inflamed mucosa results in reduction of inflammation and induction of healing in the excluded parts of the gut, while relapse occurs with restoration of fecal stream and re-exposure to luminal contents[9,10]. Similarly, ulcerative colitis patients who undergo ileal pouch-anastomosis surgery develop mucosal inflammation after bacterial colonization of the pouch[11]. A recent meta-analysis by Khan *et al*[12] has shown the significant beneficial effects of antibiotics over placebo for induction of remission in both CD and UC. Antibiotic treatment also appears to provide clinical benefits in patients with CD and inflammation of the ileal pouch[13]. Furthermore, there are many studies on animal models supporting the role of gut microbiota in the development of IBD. In experimental animal models of IBD, genetically-engineered animals developed spontaneous colitis under standard laboratory conditions, but remained colitis-free when they were raised in a sterile, germ-free environment, thus indicating that bacterial exposure and colonization are essential for the development of colitis[13-16]. Additionally, it has been shown that animal models with chemically induced colitis do not develop intestinal inflammation if they are pretreated with antibiotics[17].

The majority of genes found to be associated with an increased risk for the development of IBD are those encoding proteins that act to preserve the mucosal barrier and/or regulate the host immune system. A major breakthrough in understanding the linkage between genetic predisposition and IBD development was the discovery of the NOD2/CARD15 gene, which encodes a protein belonging to the family of pattern-recognition receptors responsible for microbial recognition, induction of antimicrobial genes, and control of the host adaptive immune response[18]. The genetic defects found in IBD CD patients might make these individuals particularly susceptible to infection by intracellular bacteria such as *Mycobacterium avium paratuberculosis*, *Listeria monocytogenes*, and adherent-invasive *Escherichia coli*[19]. Mutations in genes for toll-like receptors, as well as for the CARD4/NOD1 receptor, may also be associated with increased susceptibility for IBD[20-23].

Patients with CD have increased intestinal permeability, which could reflect mucosal barrier defects that promote bacterial translocation through the intestinal mucosa[24]. The intestinal mucus barrier is significantly altered in UC patients, particularly in terms of mucus composition and phospholipid concentration[25]. Altered function of defensins, antimicrobial peptides with bactericidal activities, might also be involved in IBD[1,24].

Despite much evidence that intestinal microorganisms are required for the triggering and perpetuation of inflammation in IBD, it still remains enigmatic whether a single specific microorganism or a group of microbial agents sharing distinctive characteristics could be responsible, or if it is actually the aberrant immune response to the dysbiosis of the commensal intestinal microbiota that plays the most major role.

Mycobacterium avium paratuberculosis used to be a particularly strong candidate as the single etiologic agent in CD in the past, since it has been shown to cause granulomatous enterocolitis in cattle that closely resembles CD in humans[26]. However, a two-year trial of combined antibiotic therapy with clarithromycin, rifabutin, and clofazimine (drugs efficient against *Mycobacteria*) did not reveal any difference in disease activity in CD patients with or without antibiotic treatment[27]. Increased numbers of invasive mucosa-associated or even intramucosal *Escherichia coli* (*E. coli*) have been reported in patients with CD and UC; a new potentially pathogenic group called adherent-invasive *E. coli* (AIEC)[20,22,28-30]. AIEC are able to adhere to and invade intestinal epithelial cells with a macropinocytosis-like process. They are capable of surviving and

replicating within macrophages, and are known to induce the release of large amounts of pro-inflammatory cytokines, such as TNF-α, by the infected host cell[23].

Although microbial pathogens have been postulated to cause Crohn's disease and ulcerative colitis since their original descriptions, it is now generally accepted that commensal enteric bacteria, either incidentally or specifically, play an important or even central role in the pathogenesis of inflammatory bowel disease, and provide the constant antigenic stimulation that continuously activates pathogenic T cells to cause chronic intestinal injury[1,5]. Four broad mechanisms have been proposed to drive pathogenic immunologic responses to luminal microbial antigens: microbial pathogens inducing intestinal inflammation, dysbiosis of commensal microbiota with a decreased ratio of protective/aggressive commensal bacterial species, host genetic defects in containing commensal microbiota, and defective host immunoregulation. These mechanisms increase exposure of bacterial antigens to mucosal T cells or alter host immune responses to commensal bacteria[5].

In normal hosts, commensal bacteria activate a sequential program of homeostatic responses by epithelial cells, macrophages, dendritic cells, and T and B lymphocytes that permit coexistence with microbes and their products[5,31,32]. In IBD, genetically predisposed individuals appear to lose the normal tolerance to commensal bacteria, leading to a chronically active inflammation process in which the microbiota provide constant stimulus for the host immune system, causing perpetuation of the disease[17]. Tissue damage might result from an immunologic misperception of indigenous flora as dangerous organisms or from the failure of normal regulatory constraints on mucosal immune responsiveness to intestinal bacteria[33]. There is growing evidence that the interplay between intestinal microbes and the mucosa of susceptible individuals triggers a cascade of reactions that starts with the interaction of microbes with specific receptors on intestinal epithelial cells, dendritic cells, and other antigen-presenting cells, followed by the interaction of these activated cells with lymphocytes, resulting in their differentiation into different subsets, driving either Th1 or Th2 inflammatory responses with the production of a wide range of inflammatory mediators, and consequently leading to mucosal damage[34]. CD is regarded to be a Th1 immune reaction driven state, whereas UC is a Th2 immune state. Bacterial recognition is dependent on transmembrane pattern recognition receptors of intestinal epithelial cells, including toll-like receptors (TLR) and the intracellular NOD-like receptor family[5,31,35,36]. Ligation of these bacterial receptors stimulates central signaling cascades that include the nuclear factor-kappaB (NFκB) pathway, one of the key pathways in mucosal homeostasis that is shown to be elevated in the chronic inflammation tissue of the IBD[5,37].

Composition of gut microbiota in patients with IBD has been extensively studied over the last decade. Although methodologies and results may differ, some generalizations are possible[38]. Numerous studies revealed that fecal microbiota has a different composition in IBD patients compared to healthy controls, and some differences between microbial populations in CD and UC were found. Similar findings were described for mucosa-associated microbiota, a bacterial population present on the mucosal surface that is in direct interaction with intestinal epithelial and immune system cells[39-42]. Moreover, differences were observed between active and non-active stages of the disease as well as between inflamed and non-inflamed regions of the intestine[41,43,44]. When studying intestinal flora in IBD, it is important to keep in mind certain facts. Firstly, only up to 30% of the total microflora can be identified using conventional bacteriological techniques[38], however using molecular techniques has greatly improved the detection rate, though significant numbers of bacteria can still be left undetected[38,45]. Secondly, many strains found in IBD do not belong to major phylogenetic groups represented in healthy individuals[38,46]. Furthermore, a distinction should be made between mucosal flora and fecal flora. The composition of these two domains is unique, which seems to be important in IBD[38,47].

Concentrations of mucosal bacteria are high in patients with bowel inflammation, especially those with CD, whereas they are low in healthy controls. Bacterial invasion of mucosa was evident in up to 83% of biopsies from IBD patients, while no bacteria were detected in tissue samples from controls[45,48]. Functional alterations are most evident in adherent, invasive *Escherichia coli* that colonize the ileum of Crohn's disease patients[49]. Fluorescent in situ hybridization studies demonstrate dramatically increased mucosa-associated bacteria in active Crohn's disease, and to a lesser extent in ulcerative colitis[48]. The fecal microbiota differs from the mucosa-associated microbiota[6], with the latter probably being more relevant for intestinal immunomodulation[48].

Reduced microbial diversity in inflammatory bowel disease has been previously reported[50-52]. Ott *et al*[50] demonstrated that mucosal inflammation in IBD was associated with a loss of normal anaerobic bacteria; the reduction in diversity in IBD was due to a significant loss of *Bacteroides*, *Eubacterium*, and *Lactobacillus* species. The reduction in mucosa-associated *Bifidobacteria* and increase in *E. coli* and *Clostridia* in patients with IBD supports the hypothesis that an imbalance between potentially beneficial and pathogenic bacteria may contribute to its pathogenesis[50,53-55]. Manichanh *et al*[52] used a metagenomic approach to demonstrate the reduced complexity of the bacterial phylum *Firmicutes*, in particular *Clostridium leptum*, in CD patients compared to healthy controls. In general, fewer *Bacteroidetes* and *Firmicutes* were found[56,57], including *Faecalibacterium prausnitzii* and bacterial species with a large butyrate-generating and anti-inflammatory capacity[39,42,57,58], as well as a reduced diversity within this phylum[59]. The counts of other short chain fatty acid (SCFA) producing bacteria such as *Bifidobacteria* are also reduced and consequently concentrations of SCFA in the intes-

tine decrease[60-62]. Other studies have shown, however, that the number of mucosa-associated bacteria increased with the increase of *Enterobacteriaceae*, including adherent-invasive *E. coli*[43,62-64].

A comprehensive study of 190 resected tissue samples by Frank *et al* showed decreased numbers of the phyla *Firmicutes* and *Bacteroidetes* with concomitant increases in *Proteobacteria* and *Actinobacteria*[59]. In a study of adult patients, Gophna *et al* compared the tissue-associated intestinal microbiota in biopsy samples from patients with CD and UC, as well as from healthy controls. Their findings showed a significant increase of *Proteobacteria* and *Bacteroidetes* in CD patients and a decrease in *Clostridia* in this group. Comparison between the ulcerative colitis and healthy control groups displayed no significant differences. Based on the finding that the microbiota was of similar composition in samples from inflamed and non-inflamed tissues within the same individual, they concluded that imbalance in microbiota in CD is probably not sufficient to cause inflammation[64].

Nwosu *et al*[65] investigated correlation of age dependency and IBD. Their findings demonstrated an apparent opposite age-related trend for *Bacteroides* and *Escherichia* between UC and CD, suggesting an immunological effect of *Bacteroides* on promoting CD at early age while later having a protective role, suggesting that these differences reflect underlying immunological disorders for CD and UC.

Up to 95% of patients with active colitis may harbor sulfate reducing bacteria (SRB)[55,66,67]. Fecal samples of patients with UC have been shown to have greater than normal levels of SRB and it has been suggested that SRB may play an important role in UC pathogenesis. Theoretically, the impairment of butyrate metabolism within colonocytes may lead to increased villous atrophy, which is one of the features of active inflammation of colonic mucosa[54].

Pediatric populations are useful for research into gut microbiota in IBD, as most pediatric patients are treatment-naïve or newly diagnosed. Although most research has been performed on adults, microbiota of pediatric IBD has been increasingly investigated over the last few years. The first larger pediatric microbiota investigation in IBD patients by Conte *et al*[29] showed a higher number of mucosa-associated facultative-anaerobic and aerobic bacteria in the ileum, cecum, and rectum of children with IBD than in controls, with the highest numbers found in patients with indeterminate colitis and Crohn's disease. They also found a good deal of individual variability in the concentrations of mucosa-associated bacteria within the different groups of patients examined, although the highest heterogeneity of species was found in the ileal mucosa of patients with Crohn's disease.

Microbial dysbiosis was also demonstrated using fecal samples in 19 children with newly diagnosed Crohn's disease. This study showed significantly lower concentrations of *Firmicutes*, mainly due to changes in detection within the *Clostridia* class, and higher concentrations of *Proteobacteria* and *Bacteroidetes*, whereas the concentration of *Actinobacteria* was similar in CD patients and controls. Furthermore, Kaakoush *et al*[68] concluded that the ratio of *Bacteroidetes* to *Firmicutes* increased with the PCDAI activity index of the patients.

Lionetti *et al*[69] suggested that a possible mechanism of action of enteral nutrition in inducing disease remission in pediatric patients with Crohn's disease is the modification capacity of the gut microbiota. This was supported by the findings that in 8 out of 9 pediatric Crohn's patients, enteral nutrition alone induced disease remission. In all children with CD, analysis of gel band distribution revealed profound modification of the fecal microflora after exclusive enteral nutrition therapy, whereas in healthy controls no modification of microflora was detected and a bacterial profile analysis remained stable during the 3-mo observation period.

Horizontal distribution of the fecal microbiota in adolescents with IBD was investigated by Gosiewski *et al*[70], who demonstrated that distribution of the microbiota in the colon is layered. Their results demonstrated that the quantitative composition of the bacterial microbiota changed in the consecutive fecal fractions and tissue samples of patients with CD and UC, whereas in the control group there were no differences in microbiota composition in consecutive fecal and tissue samples. The largest differences in the total proportion of bacteria were visible in the *Bifidobacterium* genus, whose number declined with consecutive fractions, whereas in controls it remained high in all fractions. Also, in patients with CD, the percentage of bacteria from the *Streptococcus* genus and *Enterobacteriaceae* in subsequent fractions increased in comparison to the control group, and in patients with UC similar findings were described for *Lactobacilli*. Investigation of the *Bacteroides spp.* showed that their percentage dropped in the consecutive fecal fractions in CD, similarly to the control group, whereas in patients with UC it increased. Only in the UC group was the bacterial flora attached to the mucous layer found to exert degrading action on the protective mucin[70]. Mucus layer thickness in adolescents with IBD was studied in a group by Fyderek *et al*[71]. They demonstrated that the mucus layer in the inflamed sites was significantly thinner as compared to controls and to non-inflamed sites in IBD patients. Furthermore, they reported that *Streptococcus spp.* were predominant in the inflamed mucosa in CD patients, and *Lactobacilli spp.* were predominant in UC patients.

In a study of 15 treatment-naïve pediatric patients with CD and 26 healthy controls, Kellermayer *et al*[72] investigated mucosal microbiota with high-throughput methodologies. Using distance-based redundancy analysis, they showed that there was significant separation between the CD-associated colonic mucosal microbiota and the microbiota of controls. They also showed that patients with granulomatous CD had a higher number of genera and species, significantly differentiating the colonic mucosal microbiota from controls and patients without granulomas. The most prominent genera distinguishing granulomatous CD from non-granulomatous were *Rumi-*

nococcus, *Roseburia*, *Eggerthella* (all three decreased), and *Porphyromonas* (increased). There was a trend for the genera *Faecalibacterium* to be decreased in the transverse colonic mucosa of granulomatous patients with CD compared with non-granulomatous disease[72].

A Scottish group by Hansen *et al* has been intensively investigating pediatric gut microbiota in IBD patients over the last few years. They have reported differences in colonic mucosal bacteria between pediatric UC patients and controls. Contrary to findings from previous studies, they reported a reduction in *Bacteroidetes* and an increase in *Firmicutes*[73]. They also described a reduction in bacterial diversity and an increased concentration of *Faecalibacterium prausnitzii* in de-novo pediatric CD patients, a finding contradicting the current protective role model of *F. prausnitzii* in CD[74]. In the latest study by this group, microaerophilic microbiota of pediatric IBD onset has been researched. *Campylobacter* appears to be commonly isolated from pediatric colonic biopsies, but does not seem to be strongly associated with IBD. As a common commensal in pediatric gut microbiota, *Sutterella wadsworthensis* has also been reported[75]

Despite many discoveries in the last two decades, it remains unknown whether the intestinal microbiota triggers and maintains the chronicity of inflammatory response in IBD, or is altered as a secondary response to intestinal inflammation[76].

PROBIOTICS AND PREBIOTICS

Probiotics are specific live microorganisms which, when ingested in sufficient amounts, can promote health in the host[77]. In order to qualify as probiotic, microorganisms must fulfill a number of criteria[78]. They should be strictly specified at the genus, species, and strain levels, and specific strains should be registered and disposed in an international culture collection. Thus, generalizations concerning the efficacy of a whole species or even genus might be misleading. Probiotics should be extremely safe; their safety is supported by the fact that many strains are of human origin and have a long history of safe use. Many probiotics and their applications have been granted GRAS (generally regarded as safe) status. Although this classification should not be generalized, it does not warrant permanent surveillance for potential risks, such as invasiveness and potential for transfer of antibiotic resistance to other microorganisms[79,80]. Because the effects of probiotic microorganisms are generally dependent on their viability, their stability during processing and storage, as well as their ability to survive intestinal transit through the stomach and proximal small bowel to finally adhere to mucosa and colonize the intestine, should be demonstrated[78]. The final, but perhaps one of the most important, criteria for specific microorganism to be qualified as probiotic is a scientifically proven effect on the promotion of health or prevention and treatment of a specific disease[78].

Prebiotics are non-digestible food ingredients that selectively stimulate favorable bacterial growth and/or promote activity of a limited number of health-promoting bacteria, hence benefiting the host[81,82]. However, prebiotics can also be applied to enhance the survival and action of ingested probiotic bacteria. When probiotics and prebiotics are combined in one product to achieve synergistic effects they are usually called synbiotics. The vast majority of prebiotic substances are carbohydrates that are indigestible for human digestive enzymes but can be fermented by beneficial bacterial genera in the colon and serve as a substrate for their metabolism. Some of them can be found in natural foods, such as human milk oligosaccharides in mother's milk, while others are added to food. Good examples of prebiotics are fructo-oligosaccharides (FOS), inulin, galacto-oligosaccharides (GOS), soybean oligosaccharides, and complex polysaccharides that constitute dietary fiber[81].

Probiotics or prebiotics may achieve their therapeutic effect in IBD through many different mechanisms. They influence the composition of intestinal microbiota and alter the metabolic properties of the microbiome[76]. By increasing the production of short-chain fatty acids, they may lower the pH of the colonic environment and thus inhibit the growth of potentially pathogenic microorganisms. Butyrate plays a trophic role as a nutrient for colonocytes and enhances repair of injured gut epithelium in IBD. Moreover, evidence shows that butyrate acts directly as an anti-inflammatory agent by inactivating the intracellular transcriptional factor NFκB pathway, consequently attenuating synthesis of inflammatory cytokines[8]. A large number of probiotic strains are able to produce antibacterial substances, such as hydrogen peroxide, hydrogen sulfide, lactic acid, and specific bacteriocins[83], as well as displace deleterious microbes from the luminal-mucosal interface by competing for binding sites on the epithelial cell surface or mucus layer[84,85].

Probiotics communicate with epithelial cells and different sets of cells implicated in both innate and acquired immune response via pattern-recognition receptors[3]. They can enhance gut barrier function and reduce intestinal permeability for intestinal microorganisms and other antigens[86]. For example, several strains of *Lactobacilli* can up-regulate MUC3 gene expression, resulting in increased mucus production by intestinal goblet cells[87,88]. Several probiotic strains can induce the production and secretion of different anti-microbial peptides by epithelial cells, such as defensins, lysozyme, lactoferrin, or phospholipase, and directly decrease permeability of the epithelial layer by enhancing tight junctions and reducing epithelial cell apoptosis[85,89,90].

Each probiotic strain may have distinct immunoregulatory properties, thus probiotics can indirectly or directly modulate intestinal immune response. In very simplified terms, probiotics can be classified into two groups with regards to their influence on the immune system: one exhibiting immunostimulating activities and the other anti-inflammatory properties[91]. Numerous studies have revealed the mechanisms by which probiotics down-reg-

ulate the inflammatory immune response, including those with proven clinical efficacy in the therapy of IBD. Some probiotic strains may induce maturation of intestinal dendritic cells, an important part of antigen presenting and immune regulation, and extend their survival[92]. Several probiotics act through strengthening the regulatory T cell (Treg) response. Tregs are antigen-specific T cells which prevent autoimmunity and preserve tolerance towards harmless antigens, including intestinal commensal microbiota[84]. They can control excessive NFκB pathway activation, decrease production of pro-inflammatory cytokines (*e.g.*, TNFα, INFγ, and IL-8), and induce the production and secretion of anti-inflammatory cytokines such as IL-10 and TGFβ[3,91,93,94].

It is possible that there are further mechanisms of probiotic action that have not yet been demonstrated. Regarding the fact that pathogenesis of each type of IBD differs and that mechanisms of action of probiotics are strain-specific and very different, we might expect that different probiotics would be effective for each type and phase of the disease.

Over the last two decades, several interventional clinical studies comparing the efficacy of probiotic therapy against placebo or standard therapy with drugs have been published. The use of different study designs (*e.g.*, concomitant use of other forms of therapy) and various probiotic strains and doses, with only a few studies resembling one another in such a manner to be able to uniformly compare the results, makes it very difficult to derive any firm conclusions.

TREATMENT OF ACTIVE ULCERATIVE COLITIS

Clinical studies on the efficacy of probiotics for the induction of remission in ulcerative colitis gave encouraging, albeit conflicting, results. Bennet and Brinkman first reported a successful induction of long-lasting remission by a single enema of the fecal microbiota of a healthy donor in a patient with active UC[95]. Borody *et al*[96] published six cases of patients with UC resistant to medical therapy with steroids and immunomodulators who underwent transplantation of fecal microbiota from healthy donors by repeated enemas after 7-10 d of pre-therapy with vancomycin, metronidazole, rifampicin, and bowel lavage with polyethylene glycol. Complete reversal of UC was achieved in all patients, and they were all able to stop anti-inflammatory therapy after 6 wk. After 1 to 13 years of follow-up, all patients remained in complete clinical, endoscopic, and histologic remission without any adjunctive therapy.

Several studies investigated the efficacy of multispecies probiotic VSL#3 containing four strains of *Lactobacilli* (*L. casei*, *L. plantarum*, *L. acidophilus*, and *L. delbrueckii* subsp. *bulgaricus*), three strains of *Bifidobacteria* (*B. longum*, *B. breve*, and *B. infantis*) and one strain of *Streptococcus* (*S. salivarius* subsp. *thermophilus*). Tursi *et al*[97] compared the efficacy and safety of low-dose balsalazide (2.25 g/d) plus 3 g/d VSL#3 (group A, $n = 30$), with medium-dose balsalazide alone (group B, $n = 30$), and with mesalazine (group C, $n = 30$) in the 8-wk treatment of mild to moderate active ulcerative colitis. Efficacy was assessed by assessment of symptoms, endoscopic appearance, and histological evaluation. Balsalazide/VSL#3 was significantly superior to balsalazide alone and to mesalazine in obtaining remission (85.71% *vs* 80.77% *vs* 72.73%, respectively; $P < 0.02$). The balsalazide/VSL#3 combination was faster in obtaining remission than balsalazide alone or mesalazine (4, 7.5, and 13 d, respectively), and was also better in improving all parameters evaluated. Moreover, balsalazide with or without VSL#3 was better tolerated than mesalazine. The authors concluded that balsalazide/VSL#3 might be a very good choice in the treatment of active mild-to-moderate active ulcerative colitis. Bibiloni *et al*[98] studied the efficacy and safety of VSL#3 for induction of remission in an open-label study in 34 ambulatory patients with mild to moderate active UC. Among 32 patients who completed 6-wk treatment with VSL#3 3.6×10^9 CFU/d, remission (defined as UCDAI < or = 2) was achieved in 53% and response (decrease in UCDAI > or = 3, but final score > or =3) in 24%. In 9% of patients there was no response, in another 9% worsening of the condition was observed, and in 5% there was no final endoscopic assessment. The investigators reported no biochemical or clinical adverse events related to VSL#3. In addition, they confirmed the presence of VSL#3 species by DNA sequencing of 16S rRNA in biopsies collected from patients in remission. A small open-label pilot study on 18 pediatric patients between the ages of 3-17 years with mild to moderate acute UC using VSL#3 for 8 wk was performed by Huynh *et al*[99]. The simple clinical colitis activity index (SCCAI) was used to assess disease activity. Remission (defined as SCCAI $\leqslant 3$) was achieved in 56% and response (decrease in SCCAI $\geqslant 2$, but final score $\leqslant 5$) in 6%, with no change or worsening reported in 39% of patients. Five patients were withdrawn due to lack of improvement and only 13 patients completed 8 wk of VSL#3 treatment. VSL#3 was well tolerated, and no biochemical or clinical adverse effects attributed to VSL#3 were identified.

Tursi *et al*[100] compared the efficacy of VSL#3 in a dosage of 3.6×10^9 CFU ($n = 65$) with placebo ($n = 66$) in achieving remission in UC patients on concomitant therapy with aminosalicylates and/or immunosuppressants. After 8 wk of treatment, the decrease in UCDAI of 50% or more was significantly higher in the VSL#3 group (63.1%) than in the placebo group (40.8%) ($P = 0.010$). A decrease of three points or more in the UCDAI score was achieved in 60.5% in the VSL#3 group *vs* 41.4% in the placebo group ($P = 0.017$). They also found a significant difference in rectal bleeding ($P = 0.014$) but not in stool frequency, physician's rate of disease activity, or endoscopic score. Remission was slightly higher in the VSL#3 group than in the placebo group (47.7% *vs* 32.4%; $P = 0.069$).

In a randomized, multicenter, double-blind, controlled

trial, Sood *et al*[101] compared the efficacy of VSL#3 applied twice daily in a dosage of 3.6×10^9 CFU ($n = 77$) to placebo ($n = 70$) for induction of remission of mild to moderate UC. The primary endpoint was a 50% decrease in the ulcerative colitis disease activity index (UCDAI) at 6 wk. The secondary endpoints included remission by 12 wk and reduction in total individual UCDAI parameters from baseline at 12 wk. At week 6, the percentage of patients with an improvement in UCDAI score that was greater than 50% was significantly higher in the group given VSL#3 (32.5%) than the group given placebo (10%) ($P = 0.001$). At week 12, 42.9% patients given VSL#3 achieved remission, compared with only 15.7% patients given placebo ($P < 0.001$). Furthermore, significantly more patients given VSL#3 (51.9%) achieved a decrease in their UCDAI that was greater than 3 points, compared with those given placebo (18.6%) ($P < 0.001$). The VSL#3 group had significantly greater decreases in UCDAI scores and individual symptoms at weeks 6 and 12 compared with the placebo group.

Miele *et al*[102] performed a 1-year prospective, placebo-controlled, double-blind pediatric study to assess the efficacy of VSL#3 on the induction and maintenance of remission in children with active UC. A total of 29 consecutive patients (mean age: 9.8 years; range: 1.7-16.1 years) with newly diagnosed UC were randomized to receive either a weight-based dose of VSL#3 ($n = 14$) or placebo ($n = 15$) in conjunction with concomitant steroid induction and mesalamine maintenance treatment. The Lichtiger colitis activity index and a physician's global assessment were used to measure disease activity. At baseline (within 6 mo, 12 mo, or at the time of relapse), all patients were assessed endoscopically and histologically. All 29 patients responded to the induction therapy. Remission was achieved in 92.8% children treated with VSL#3 and standard therapy compared to only 36.4% treated with placebo and standard therapy ($P < 0.001$). Moreover, only 21.4% patients treated with VSL#3 relapsed within 1 year of follow-up compared to 73.3% patients from the placebo group ($P = 0.014$). At 6 mo, 12 mo, or at time of relapse, endoscopic and histological scores were significantly lower in the VSL#3 group than in the placebo group ($P < 0.05$). There were no biochemical or clinical adverse events related to VSL#3. This study demonstrated the efficacy of VSL#3 both in the induction and maintenance of remission in pediatric UC patients.

In a small open-label study by Tsuda *et al*[103], the effectiveness of another multispecies probiotic preparation BIO-THREE (containing *Streptococcus faecalis*, *Clostridium butyricum*, and *Bacillus mesentericus*) was tested for treatment of mild to moderate distal UC refractory to conventional therapies. Twenty patients were treated for 4 wk. Clinical symptoms and endoscopic findings were evaluated, and UCDAI scores calculated before and after treatment. In addition, fecal microbiota was analyzed by the terminal restriction fragment length polymorphism (T-RFLP) method. Remission (UCDAI score $\leqslant 2$) was observed in 45% and response (decrease in UCDAI $\geqslant 3$, but final score $\geqslant 3$) in 10%, however in 40% there was no response and in 5% they found worsening (UCDAI > 3) of the disease. T-RFLP analysis indicated an increase in *Bifidobacteria*.

In a single-center, randomized, double-dummy study, Rembacken *et al*[104] examined whether the addition of a non-pathogenic strain of *E. coli* Nissle 1917 to standard medical therapy increased the chance of remission of active ulcerative colitis and whether this probiotic strain was as effective as mesalazine in preventing relapse. Of a total of 116 patients, 59 were randomized to the mesalazine group and 57 to the *E. coli* group. All patients received concomitant standard medical therapy with tapering steroids together with a 1-wk course of oral gentamicin. After remission, patients were maintained on either mesalazine or *E. coli*, and followed-up for 1 year. The investigators found no significant differences between the mesalazine and *E. coli* groups in percentage of patients that achieved remission, mean time to remission, percentage of patients who relapsed, and mean duration of remission. Although the addition of *E. coli* to standard therapy did not increase the induction rate of remission, the results suggested that treatment with this probiotic might have an equivalent effect to mesalazine in maintaining remission of ulcerative colitis.

Kato *et al*[105] conducted a randomized placebo-controlled trial using *Bifidobacteria*-fermented milk (BFM) (containing *Bifidobacterium breve* strain Yakult, *B. bifidum*, and *Lactobacillus acidophilus*) supplementation as a dietary adjunct in treating active ulcerative colitis. Twenty patients with mild to moderate active UC randomly received 100 mL/d of BFM or placebo for 12 wk with conventional treatment. The clinical activity index was significantly lower in the BFM than in the placebo group, and the endoscopic activity index and histological score were significantly reduced in the BFM, but not the placebo group, after treatment. They also observed an increase in fecal butyrate, propionate, and short-chain fatty acid concentrations in the BFM, but not the placebo group. Therefore, the authors concluded that supplementation with this *Bifidobacteria*-fermented milk product is safer and more effective than conventional treatment of active UC alone.

Ishikawa *et al*[106] compared a group of patients with BFM supplementation 100 mL/d ($n = 11$) and a control group ($n = 10$), both receiving standard medical treatment of ulcerative colitis. Colonoscopies, general blood markers, and examinations of intestinal flora, including the analysis of fecal organic acids, were performed at the initiation of the study and after one year. Exacerbation of symptoms was observed in 3 out of 11 subjects in the BFM group and in 9 out of 10 in the control group. Statistical analysis of the cumulative exacerbation rates showed a significant reduction in exacerbations for the BFM group ($P = 0.0184$). A significant reduction in the relative proportion of *B. vulgatus* in *Bacteroidaceae* and butyrate concentration was observed after supplementation

with BFM in comparison with before.

Recently, Oliva *et al*[107] published a prospective, randomized, placebo-controlled study comparing the effectiveness of *Lactobacillus reuteri* ATCC 55730 enema and placebo in children with active distal UC. A total of 40 patients (median age 7.2 years; range 6-18 years) were enrolled. They received an enema solution containing 10^{10} CFU of *L. reuteri* or placebo for 8 wk, in addition to oral mesalazine. Clinical, endoscopic, and histological scores, as well as rectal mucosal expression levels of pro- and anti-inflammatory cytokines, were evaluated at the beginning and at the end of the trial. Mayo score (including clinical and endoscopic features) as well as histological score decreased significantly in the *L. reuteri* group ($P < 0.01$), but not in the placebo group. Moreover, the evaluation of cytokine mucosal expression levels revealed that IL-10 significantly increased ($P < 0.01$), whereas IL-1β, TNFα, and IL-8 significantly decreased ($P < 0.01$) only in the *L. reuteri* group.

In a small non-controlled pilot study, Guslandi *et al*[108] treated 25 patients with mild to moderate clinical flare-up of ulcerative colitis with *Saccharomyces boulardii* 250 mg three times a day for 4 wk during maintenance treatment with mesalazine. Of the 24 patients who completed the study, 17 attained clinical and endoscopic remission.

Furrie *et al*[109] explored the efficacy of a synbiotic combining a probiotic strain of B*ifidobacterium longum* and a prebiotic (Synergy 1), a preferential inulin-oligofructose growth substrate for this probiotic strain. Treatment was used in a double-blinded randomized controlled trial in 18 patients with active UC for a period of one month. Although the subsequent sigmoidoscopy score decrease in the synbiotic group was not statistically significant compared with placebo ($P = 0.06$), they found that biopsies in the test group had reduced inflammation, and increased regeneration of epithelial tissue and mRNA levels for beta defensins 2, 3 and 4 (which are strongly up-regulated in active UC), tumor necrosis factor alpha and interleukin-1 alpha were also significantly reduced in the test group after treatment ($P = 0.016$, 0.038, 0.008, 0.018 and 0.023, respectively).

In another study by Ishikawa *et al*[110], the investigators examined the effects of a live *Bifidobacterium breve* strain Yakult and GOS as synbiotic in active UC. Forty-one patients with mild to moderate UC were assigned to two groups; one was treated with the synbiotic (1 g of the probiotic powder (10^9 CFU/g) three times a day and 5.5 g of GOS once a day) and the other was not (control group). After one-year treatment with the synbiotic, the clinical status of the UC patients as assessed by colonoscopy significantly improved, and the amount of myeloperoxidase in the lavage, a marker of inflammation, decreased. The synbiotic also significantly reduced the fecal counts of *Bacteroidaceae* and fecal pH.

Several reviews and meta-analyses have been performed over recent years concerning the induction of remission in ulcerative colitis by probiotics. In a Cochrane Collaboration review from 2007, the authors assessed the efficacy of probiotics compared to placebo or standard medical treatment with 5-aminosalicylates, sulfasalazine, or corticosteroids[111]. Only 4 randomized controlled trials met the criteria, and a formal meta-analysis could not be performed because of heterogeneity in methodology, probiotic strains, and outcomes. The authors concluded that combining conventional therapy with probiotics did not improve overall remission rates in patients with mild to moderate UC. However, they found limited evidence that the addition of probiotics might provide modest benefits in terms of disease activity. The negativistic opinion shared in this early review can be at least partially attributed to the low number of high quality studies published at the time. In a meta-analysis later performed by Sang *et al*[112] and published in 2010, both the induction of remission and maintenance were compared between probiotic and non-probiotic treatment in ulcerative colitis. Thirteen randomized controlled studies met the selection criteria. Seven studies evaluated the remission rate, 8 the recurrence rate, and 2 both remission and recurrence rates. The remission rate for probiotics compared with non-probiotics therapy was 1.35 (95%CI: 0.98-1.85), while when compared with the placebo it was 2.00 (95%CI: 1.35-2.96). Although these differences were not statistically significant, the authors concluded that these results were probably subject to heterogeneous bias. Regarding maintenance of remission, the recurrence rate of ulcerative colitis in patients who received probiotics was 0.69 (95%CI: 2.47-1.01) and 0.25 (95%CI: 0.12-0.51) in patients with mild to moderate UC compared with the non-probiotic group. The group who received *Bifidobacterium bifidum* treatment had a recurrence rate of 0.25 (95%CI: 0.12-0.50) compared with the non-probiotics group. The authors concluded that probiotic treatment was more effective than placebo in maintaining remission in ulcerative colitis.

In contrast with these reviews, a meta-analysis performed by Zigra *et al*[113] showed a significant benefit of probiotic use for UC remission induction with pooled relative risk 2.27 (95%CI: 1.00-5.14, $P = 0.049$).

In a more recent review by Jonkers *et al*[56], only subgroup-specific meta-analyses per probiotic were performed. The only probiotic with several published randomized controlled studies for induction of remission in adult patients with UC was VSL#3. The calculated pooled RR for VSL#3 was 1.69 (95%CI: 1.17-2.43), indicating a significant benefit of VSL#3 over control in inducing remission in active UC.

Interestingly, in the 2011 recommendations for probiotic use from the 3rd Yale Workshop, both VSL#3 and *Escherichia coli* Nissle 1017 were rated B, meaning that recommendation of their use for induction of remission in UC is based on positive controlled studies, but with the presence of some negative studies that did not support the primary outcome[114].

In conclusion, the results of several clinical studies suggest that the addition of specific probiotics to conventional therapy in active UC may be beneficial. The

strongest evidence exists for multispecies preparation VSL#3, with several studies both in adults and children supporting its efficacy.

MAINTENANCE OF REMISSION IN ULCERATIVE COLITIS

There have been several published studies in which efficacy of the probiotic strain of *Escherichia coli* Nissle 1917 was compared to either placebo or standard therapy for maintenance therapy in UC. In a double-blind, double-dummy study by Kruis *et al*[115], 120 patients with inactive ulcerative colitis were randomized to mesalazine 500 mg three times daily or to an oral preparation of *E. coli* Nissle treatment for 12 wk to compare their efficacy in preventing a relapse of the disease. Study objectives were to assess the equivalence of the two therapeutic modalities by comparing the clinical activity index (CAI), relapse rates, relapse-free times, and global assessment. The start and end CAI scores demonstrated no significant difference ($P = 0.12$) between the two treatment groups. Relapse rates were 11.3% under mesalazine and 16.0% under *E. coli* (N.S.), and the relapse-free time was similar for mesalazine and *E. coli* (103 +/- 4 d and 106 +/- 5 d, respectively). Global assessment was also similar for both groups. Tolerability of the treatment was excellent in both groups. Conclusions of this study were that probiotic treatment with *E. coli* Nissle 1917 offered another option for maintenance therapy of ulcerative colitis. Subsequently, the same group performed another, albeit larger, double-blind, double dummy trial to confirm the equivalent efficacy of *Escherichia coli* Nissle 1917 and mesalazine in the maintenance of remission in UC[116]. Patients received either the probiotic drug 200 mg once daily ($n = 162$) or mesalazine 500 mg three times daily ($n = 165$) for 12 mo, and were assessed by clinical and endoscopic activity indices (Rachmilewitz) as well as by histology. The per-protocol analysis revealed relapses in 40/110 (36.4%) patients in the *E. coli* group and 38/112 (33.9%) in the mesalazine group (significant equivalence $P = 0.003$). Subgroup analyses showed no differences between the treatment groups in terms of duration and localization of disease or pretrial treatment. Safety profile and tolerability were very good for both groups. By the end of this second study the authors concluded that *E. coli* Nissle 1917 showed the same equivalent efficacy and safety as mesalazine in maintaining remission in patients with ulcerative colitis.

In another trial by Rembacken *et al*[104], both the capacity of induction and maintenance of remission by *E. coli* Nissle 1917 were evaluated. In this single-center, randomized, double-dummy study, patients were maintained on either mesalazine ($n = 59$) or *E. coli* ($n = 57$) and followed up for a maximum of 12 mo. A comparable percentage of patients relapsed in the mesalazine (73%) and *E. coli* groups (67%), and the mean duration of remission was practically similar in both (206 and 221 d, respectively). Again, the authors came to the conclusion that treatment with non-pathogenic *E. coli* was as equivalently efficient as mesalazine in maintaining remission of ulcerative colitis.

Zocco *et al*[117] studied the efficacy of a probiotic strain of *Lactobacillus rhamnosus* GG for maintenance therapy in UC. They randomized patients into three groups: *Lactobacillus* GG 18×10^9 CFU/d ($n = 65$), mesalazine 2400 mg/d ($n = 60$), or a combination of *Lactobacillus* GG and mesalazine ($n = 62$). Overall analysis of UCDAI scores and endoscopy and histology results showed no difference in relapse rate at 6 and 12 mo among the three groups. However, treatment with *Lactobacillus* GG alone or in combination seemed to be more effective than standard treatment with mesalazine in prolonging relapse-free time ($P < 0.05$).

A non-controlled trial using multispecies preparation VSL#3 in 20 UC patients in remission, intolerant, or allergic to 5-aminosalicylates for 12 mo was performed by Venturi *et al*[118]. They reported that 15 out of 20 patients remained in remission during the study, 4 relapsed, and one was lost to follow-up. They suggested that VSL#3 might be useful in maintaining remission in UC patients intolerant to standard therapy.

In the previously mentioned pediatric study by Miele *et al*[102], the investigators observed that only 21.4% of patients treated with VSL#3 (compared to 73.3% patients from the placebo group) relapsed within 1 year of follow-up ($P = 0.014$). They also found significantly lower endoscopic and histological scores in the VSL#3 group than in the placebo group ($P < 0.05$). The results of this study confirmed the efficacy of VSL#3 in the maintenance of remission in pediatric UC patients.

Cui *et al*[119] randomized 30 patients with UC in remission achieved by treatment with sulfasalazine and glucocorticoids into two groups: one that received bifid triple viable capsule (BIFICO) (1.26 g/d) for 8 wk and the other an identical placebo group. The patients were evaluated clinically, endoscopically, and histologically after 2 mo of treatment or in the event of UC relapse. Only three patients (20%) in the BIFICO group relapsed during the 2-mo follow-up period compared with 14 (93.3%) the in placebo group ($P < 0.01$). Moreover, the microbiological and immunological analyses revealed that the concentration of fecal *Lactobacilli* and *Bifidobacteria* was significantly increased only in the BIFICO-treated group ($P < 0.01$). The expression of pro-inflammatory NFκB p65 and DNA binding activity of NFκB were significantly attenuated, and the mRNA expression of anti-inflammatory cytokines was elevated in the treatment group in comparison with the control group ($P < 0.05$). The authors concluded that oral administration of probiotic preparation BIFICO was effective in preventing flare-ups of chronic UC.

Shanahan *et al*[120] performed a double-blind, placebo-controlled study on 157 patients to compare the efficacy of *Lactobacillus salivarius* subspecies *salivarius* UCC118, *Bifidobacterium infantis* 35624 (1×10^9 CFU/d), or placebo for maintenance UC therapy. They found no difference

in relapse time between probiotics and placebo.

Wildt *et al*[121] performed a small double-blind placebo-controlled study using probiotic preparation Probio-Tec-AB-25 (containing the two probiotic strains *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* subspecies *lactis* BB-12) or placebo in patients with left-sided UC in remission for 52 wk. 25% of patients on probiotics and 8% of those on placebo maintained remission after 1 year of treatment ($P = 0.37$). The median time to relapse was 125 d in the probiotic group and 104 d in the placebo group ($P = 0.683$). The authors concluded that no significant clinical benefit of Probio-Tec-AB-25 in comparison with placebo for maintaining remission in UC was demonstrated.

In the recent Cochrane Collaboration review of probiotic efficacy and safety for the maintenance of remission in UC by Naidoo *et al*[122], only 4 studies met the inclusion criteria. Three of those trials compared probiotics to mesalazine, and one to placebo. The pooled analysis was performed and revealed no statistically significant differences in the efficacy of probiotics over mesalazine. Relapse was reported in 40.1% of patients treated with probiotics and in 34.1% of those on mesalazine therapy. No statistical difference in the incidence of adverse events between the two groups was demonstrated. In only one placebo-controlled study was the relapse rate between probiotic and placebo groups considered non-significant. The authors concluded that, given the relatively small number of patients included in the clinical studies, the evidence was insufficient to make conclusions about the efficacy of probiotics for the maintenance of remission in UC.

A subgroup probiotic-specific meta-analysis by Jonkers *et al*[56] revealed that pooled relative risk for *E. coli* Nissle compared to mesalazine was 1.08 (95% CI 0.86-1.37), indicating that this strain of *E. coli* was not inferior to mesalazine in preventing relapses.

The American Recommendations for probiotic use from 2011 state very strong "A" recommendations for the use of the two specific probiotics *Escherichia coli* Nissle 1917 and multispecies mixture VSL#3 for the maintenance of remission in UC[114].

In conclusion, specific probiotics such as *Escherichia coli* Nissle 1917 and multispecies mixture VSL#3 are probably as efficient as standard maintenance therapy with mesalazine, and can therefore be used instead of mesalazine in patients intolerant or allergic to 5-aminosalicylates, or as adjunctive therapy to standard therapy, to potentially increase the duration of remission.

TREATMENT AND PREVENTION OF POUCHITIS

In some patients with UC in whom the disease does not respond to medical therapy or who develop dysplasia or cancer, proctocolectomy with the construction of ileal pouch-anal anastomosis (IPAA) is required. Inflammation of this ileal reservoir (pouch), referred to as pouchitis, develops in between 15% and 50% of such patients. Although the exact etiology of pouchitis is not clear, host genetic factors, fecal stasis, mucosal ischemia, and bacterial dysbiosis in the pouch seem to be involved[56,87]. Most patients develop pouchitis in the first year after the procedure. Antibiotic therapy is generally successful; however, discontinuation of antibiotics is often followed by recurrence of the disease. Treatment and prevention of pouchitis with probiotics has thus been studied extensively, and only a few studies addressing the use of probiotics for the treatment of active pouchitis were published.

Kuisma *et al*[123] performed a double-blind placebo-controlled trial to investigate the efficacy of *Lactobacillus rhamnosus* GG supplementation as primary therapy for ileal pouch inflammation. Twenty patients with a previous history of pouchitis and endoscopic evidence of inflammation were randomized to *Lactobacillus* GG 0.5-1 $\times 10^{10}$ CFU twice daily or placebo for 3 mo. Clinical efficacy was assessed by a change in the pouchitis disease activity index (PDAI). In addition, quantitative bacterial cultures of fecal samples and biopsies taken from the pouch were performed before and after probiotic supplementation. No differences were observed between the groups with regard to the mean pouchitis disease activity index. *Lactobacillus* GG supplementation changed the pouch intestinal microbiota by increasing the ratio of total fecal *Lactobacilli* to total fecal anaerobes ($P = 0.03$) and enhancing the frequency of *Lactobacilli*-positive cultures in the pouch. The authors concluded that although probiotic supplementation with *Lactobacillus* GG changed pouch microbiota, it was clinically ineffective as primary therapy for active pouchitis.

In an open-label study, Laake *et al*[124] treated 51 UC patients with IPAA, 6 UC patients with ileorectal anastomosis without pouch, and 10 patients with IPPA because of familial adenomatous polyposis with a fermented milk product culture, containing probiotic strains *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* subspecies *lactis* BB-12, in a dosage of 5×10^{10} CFU/d for 4 wk. Stool samples were cultured for examination of *Lactobacilli*, *Bifidobacteriae*, fungi, and pH before, during, and after intervention. In addition, before, during, and after intervention, symptom assessment and endoscopic evaluation was performed. Symptoms, such as involuntary defecation, leakage, abdominal cramps, fecal number and consistency, mucus, and urge to evacuate stools were significantly decreased during intervention in the UC/IPAA group. The median endoscopic score of inflammation also significantly decreased. The number of *Lactobacilli* and *Bifidobacteriae* significantly increased during intervention and remained significantly increased one week after intervention.

Gionchetti *et al*[125] evaluated the efficacy of high-dose VSL#3 in the treatment of mild active pouchitis in an open-label non-controlled study. Twenty-three patients with mild pouchitis were treated with VSL#3 (3.6×10^9 CFU/d) for four weeks. Symptomatic, endoscopic, and histologic evaluations were undertaken before and after treatment according to PDAI. Remission was defined as

a combination of a PDAI clinical score of ⩽ 2, an endoscopic score of ⩽ 1, and a total PDAI score of ⩽ 4. Patients in remission after initial treatment were treated with a maintenance dose of VSL#3 (1.8 10^9 CFU /d) for an additional six months. Sixteen out of 23 patients (69%) were in remission after treatment. The median total PDAI scores before and after therapy were 10 (range, 9-12) and 4 (range, 2-11), respectively ($P < 0.01$). The median Inflammatory Bowel Disease Questionnaire score also significantly improved ($P < 0.001$). All 16 patients who went into remission maintained remission during maintenance treatment. The authors conclude that high doses of the probiotic VSL#3 were effective in the treatment of mild pouchitis. As pouchitis is a recurrent state, many studies evaluated the potential of probiotics in preventing exacerbations. The most profoundly studied probiotic for this indication was multispecies preparation VSL#3. In a randomized, double-blind, placebo-controlled trial, the same group evaluated the efficacy of VSL#3 in the remission maintenance of chronic pouchitis compared with placebo[126]. Forty patients in clinical and endoscopic remission achieved by antibiotic therapy were randomized to receive either VSL#3 3 × 10^{12} CFU/d or placebo for 9 mo. Patients were assessed clinically every month, and endoscopically and histologically every 2 mo or in the event of relapse. In addition, bacterial stool cultures from fecal samples were performed before and after antibiotic treatment and each month during maintenance treatment. Only 3 patients (15%) in the VSL#3 group had relapses within the 9-mo follow-up period, in comparison with 20 (100%) in the placebo group ($P < 0.001$). In the VSL#3-treated group (but not in the control group), fecal concentrations of *Lactobacilli*, *Bifidobacteria*, and *S. thermophilus* increased significantly from baseline levels during treatment ($P < 0.01$). Therefore, the authors concluded that oral administration of VSL#3 is effective in preventing flare-ups of chronic pouchitis.

In another double-blind, placebo-controlled study, Gionchetti *et al*[127] evaluated the effectiveness of VSL#3 therapy in preventing the onset of pouchitis immediately and during the first year after ileal pouch-anal anastomosis. For this purpose, 40 patients who underwent IPAA for UC were randomized to receive either VSL#3 9 × 10^{11} CFU/d ($n = 20$) or placebo ($n = 20$) immediately after ileostomy closure for 1 year. The patients were assessed clinically, endoscopically, and histologically every few months, and health-related quality of life was assessed using the Inflammatory Bowel Disease Questionnaire (IBDQ). Only 2 (10%) patients from the VSL#3 group, compared to 8 (40%) from the placebo group, had an episode of acute pouchitis (log-rank test, $Z = 2.273$; $P < 0.05$). As expected, treatment with VSL#3 (but not placebo) produced a significant improvement in IBDQ score.

In a study by Mimura *et al*[128], the researchers evaluated the effectiveness of a single daily high dose probiotic preparation of VSL#3 in maintaining antibiotic-induced remission. All patients included in this study had pouchitis at least twice in the previous year or required treatment with continuous antibiotics. After remission was induced within four weeks of combined metronidazole and ciprofloxacin therapy, the patients were randomized to receive either VSL#3 (9 × 10^{11} CFU) ($n = 20$) or placebo ($n = 16$) once daily for one year or until relapse. Symptomatic, endoscopic, and histological evaluations were made before and 2 and 12 mo after randomization or at the time of relapse. Remission was maintained for one year in 17 patients (85%) on VSL#3, but in only one (6%) on placebo ($P < 0.0001$). The IBDQ score remained high in the VSL#3 group but deteriorated in the placebo group ($P = 0.0005$). Therefore, the authors concluded that the once daily high dose probiotic VSL#3 was effective in maintaining antibiotic introduced remission in patients with recurrent or refractory pouchitis.

In an open-label trial by Pronio *et al*[129], 31 patients at different periods after surgery without signs or symptoms of pouchitis were randomized to VSL#3 9 × 10^{11} CFU/d or no treatment for 12 mo. Pouchitis activity was evaluated by PDAI, with different immunologic parameters being studied in peripheral-blood mononuclear cells and mucosal biopsies to reveal the mechanisms of probiotic action. During the study period, none of the patients from the probiotic group and only one from the placebo group developed active pouchitis. Because of the extremely low relapse-rate, even in the non-treated group, it was impossible to derive any firm conclusions regarding the efficacy of probiotic treatment from this study. However, a significant reduction in PDAI score was observed in VSL#3 treated patients.

In contrast with these studies, Shen *et al*[130] reported much more disappointing results. In an open-label uncontrolled trial, they gave VSL#3 9 × 10^{11} CFU/d for 8 mo to 31 patients after being treated for pouchitis with ciprofloxacin for 2 wk. Baseline PDAI scores were calculated and patient symptoms were reassessed at week 3 of VSL#3 therapy and at the end of the 8-mo trial. Some, but not all, patients underwent repeat pouch endoscopy at the end of the trial. At the 8-mo follow-up, only 6 patients were still on VSL#3 therapy while all others had discontinued the therapy due to either recurrence of symptoms ($n = 23$) or development of adverse effects ($n = 2$). All six patients who completed the 8-mo course had repeat clinical and endoscopic evaluation. Their mean PDAI scores were not statistically different to those before probiotic intervention ($P = 0.27$). However, this trial had several methodological drawbacks. The patients were pre-treated with only one antibiotic and the success of this therapy of acute pouchitis was not regularly evaluated by endoscopy. Therefore, it remains unclear whether all patients were really in remission before the start of maintenance therapy with VSL#3.

In an open-label study by Gosselink *et al*[131], 39 patients given a fermented milk product containing *Lactobacillus rhamnosus* GG in a dosage of 1-2 × 10^{10} CFU immediately after IPAA operation were compared to 78 patients without any maintenance treatment. The first

episodes of pouchitis were observed significantly less frequently in the *Lactobacillus* GG group than in the untreated group (cumulative risk at 3 years: 7% *vs* 29%, $P = 0.011$). Therefore, the authors concluded that daily intake of the fermented product containing *Lactobacillus* GG provided significant clinical benefits without side effects, and recommended its use for the primary prevention of pouchitis.

In the Cochrane Collaboration review by Holubar *et al*[132] published in 2010, different modalities for the treatment and prevention of pouchitis after ileal pouch-anal anastomosis for UC, including different antibiotics, probiotics, glutamine, butyrate, and budesonide, were meta-analyzed and reviewed. They concluded that *Lactobacillus* GG was not superior in effectiveness compared to placebo for the treatment of acute pouchitis, while VSL#3 was more effective than placebo in the maintenance therapy of chronic pouchitis (97% *vs* 3%, $P < 0.0001$). The number needed to treat with VSL#3 to prevent one additional relapse was 2. Similarly, in a strain-specific meta-analysis performed by Jonkers *et al*[56], the authors calculated the pooled relative risk for prevention of relapses of pouchitis for VSL#3 compared to placebo as 0.17 (95%CI: 0.09-0.33). As a result of these conclusions, the multispecies probiotic preparation VSL#3 was granted the A level recommendation for the primary prevention and maintenance of remission of pouchitis after IPAA according to the American Recommendations for probiotic use from 2011[115]. Furthermore, they suggested that there was some evidence (C level) supporting its use even for the therapy of active pouchitis.

Finally, clinical guidelines for the management of pouchitis from 2009 suggest the use of VSL#3 in patients with recurrence of pouchitis following antibiotic treatment or having several recurrences despite antibiotic therapy[133]. However, they do not suggest probiotics for the treatment of acute pouchitis.

TREATMENT OF ACTIVE CROHN'S DISEASE

Clinical studies investigating the treatment of active Crohn's disease with probiotics were scarce. Gupta *et al*[134] reported a very small open-label pilot study of four children with mildly to moderately active Crohn's disease who were treated with entero-coated tablets containing *Lactobacillus rhamnosus* GG (10^{10} CFU) twice daily for 6 mo. Clinical activity was monitored by pediatric Crohn's disease activity index (PCDAI) and changes in intestinal permeability were measured by a double sugar permeability test. A significant improvement in clinical activity was observed 1 wk after starting *Lactobacillus* GG. Median PCDAI scores at 4 wk were 73% lower than baseline. Intestinal permeability improved in an almost parallel fashion. The authors concluded that the findings of this pilot study showed that *Lactobacillus* GG might improve clinical status and gut barrier function in children with mildly to moderately active Crohn's disease. Schultz *et al*[135] performed a small randomized, placebo-controlled trial to determine the efficacy of *Lactobacillus rhamnosus* GG in the induction or maintenance of medically-induced remission. Eleven patients with moderate to active Crohn's disease were enrolled to receive either *Lactobacillus* GG (2×10^9 CFU/d) or placebo for six months. In all patients, a tapering steroid regimen was applied for the induction of remission, and all received antibiotics the week before probiotic/placebo intervention was initiated. The primary end point was sustained remission; defined as freedom from relapse after 6 mo. Only 5 patients finished the study, with 2 patients in each group in sustained remission. The median time to relapse was 16 +/- 4 wk in the probiotic and 12 +/- 4.3 wk in the placebo group ($P = 0.5$). In contrast with the results of Gupta *et al*[134], this study did not demonstrate any benefit of *Lactobacillus* GG in inducing or maintaining medically-induced remission in CD.

Although Butterworth *et al*[136] in the Cochrane Collaboration review concluded that there was insufficient evidence to make any conclusions about the efficacy of probiotics in inducing remission in CD because of a lack of well-designed clinical studies, the two studies using synbiotics that were not included in this review revealed very promising results[137,138]. Fujimori *et al*[137] performed an open-label uncontrolled trial using a synbiotic for the therapy of active refractory Crohn's disease. Ten active CD patients who had failed to achieve remission via an initial therapeutic regimen of aminosalicylates and prednisolone were given synbiotic therapy consisting of two probiotic preparations that both contained *Bifidobacterium breve* 3×10^{10} CFU, *Lactobacillus casei* 3×10^{10} CFU, and *Bifidobacterium longum* 1.5×10^{10} CFU, as well as a prebiotic comprised of 3.3-9.9 g of psyllium (*Plantago ovata*). Patients were free to adjust their intake of probiotics or prebiotics throughout the trial. For the assessment of disease activity, Crohn's disease activity index (CDAI), International Organization for the Study of Inflammatory Bowel Disease (IOIBD) score, and blood sample variables were evaluated and compared before and after the trial. The duration of the trial was 13.0 +/- 4.5 mo. Of the ten included patients, 6 had a complete response, one had a partial response, and three were non-responders. Two patients discontinued treatment and four decreased their corticosteroid therapy. Both CDAI and IOIBD scores were significantly reduced after therapy (255-136, $P = 0.009$; 3.5-2.1, $P = 0.03$, respectively), however the laboratory markers of inflammation did not change. With the exception of some abdominal bloating disappearing with discontinuation of psyllium ingestion, there were no adverse events. The authors concluded that a combination of high-dose probiotics and prebiotics could be safely and effectively used as a co-therapy for the treatment of active CD.

Recently, Steed *et al*[138] conducted a randomized, double-blind, placebo-controlled trial including 35 patients with active CD using a synbiotic therapy comprised of probiotic *Bifidobacterium longum* 4×10^{11} CFU and prebiot-

ic Synergy 1 (oligofructose and inulin) 12g daily. Patients were requested to continue on stable doses of conventional medication they were receiving at initiation of the trial. The patients' clinical status was scored by CDAI and endoscopies with biopsies were performed at the start, and at 3 and 6 mo of therapeutic intervention. Six patients from the synbiotic group and 5 from the placebo group were lost from follow-up. Upon comparing pre- and post-treatment CDAI, there was a significant clinical improvement in the synbiotic group (start 219 ± 74.6, finish 147 ± 74; $P = 0.020$) but not in the placebo group (start 249 ± 79.4, finish 233 ± 155; $P = 0.81$). Similarly, there was a significant improvement in mean histological scores in the synbiotic group (start 6 ± 5, finish 3 ± 4; $P = 0.018$) but not in the placebo group (start 6 ± 5, finish 5 ± 6; $P = 0.75$). A significant reduction of pro-inflammatory TNF-α and an increase of mucosal *Bifidobacteria* was also observed in the synbiotic group.

MAINTENANCE OF REMISSION IN CROHN'S DISEASE

Only a few high-quality studies have been performed to assess the efficacy of probiotics for the maintenance of remission achieved with standard medical therapy or surgical resection in Crohn's disease. Currently, the use of probiotics for the maintenance of remission in Crohn's disease is not recommended.

In a trial by Guslandi *et al*[139], 32 patients with Crohn's disease in clinical remission (CDAI < 150) were randomized to treatment with either mesalamine 1 g three times daily or mesalamine 1 g two times daily plus probiotic yeast *Saccharomyces boulardii* 1 g daily for six months. Clinical relapses were observed in 37.5% of patients receiving mesalamine alone but in only 6.25% of patients combining mesalamine with the probiotic ($P = 0.04$). The authors hence concluded that *Saccharomyces boulardii* might be useful in the maintenance treatment of Crohn's disease.

Bousvaros *et al*[140] conducted a randomized, placebo-controlled trial of the probiotic *Lactobacillus rhamnosus* GG (LGG) to see whether the addition of LGG to standard therapy prolonged remission in children with CD. Seventy-five children and adolescents from 5 to 21 years old with CD in remission were randomized to receive either LGG ($n = 39$) or placebo ($n = 36$), and followed for up to 2 years. Concomitant medications, including aminosalicylates, 6-mercaptopurine, azathioprine, and low-dose alternate day corticosteroids were allowed. The percentage of patients that relapsed did not significantly differ between the LGG and the placebo group (31% *vs* 17%; $P = 0.18$), neither did the median time to relapse (9.8 mo *vs* 11.0 mo; $P = 0.24$). In conclusion, LGG did not prove to be effective for maintaining remission in children with CD when given as an adjunct to standard therapy. The ineffectiveness of probiotic strain *Lactobacillus rhamnosus* GG for maintenance therapy in CD was also supported by a study by Prantera *et al*[141], who performed a randomized placebo-controlled study in patients operated for Crohn's disease in whom all of the diseased gut had been removed. The patients received 1.2×10^9 CFU of *Lactobacillus* GG or placebo for one year. Ileocolonoscopy was performed at the end of the trial or at the onset of symptoms. Clinical recurrence was ascertained in 16.6% in the LGG group and in 10.5% in the placebo group. Sixty percent of patients in clinical remission on LGG had endoscopic recurrence compared with 35.3% on placebo ($P = 0.297$). There were no significant differences in the severity of the lesions between the two groups. Marteau *et al*[142] studied the potential of *Lactobacillus johnsonii* LA1 for prevention of recurrence in operated CD patients. This was a randomized, double-blind, placebo-controlled study. Patients were randomized to receive *Lactobacillus johnsonii* LA1 4×10^9 CFU/d ($n = 48$) or placebo ($n = 50$) for six months. No other treatment was allowed. There were 4 clinical recurrences in the probiotic group and 3 in the placebo group. In patients with symptomatic remission, endoscopic recurrence was observed in 64% in the placebo group compared to 49% in the probiotic group ($P = 0.15$). Endoscopic score distribution did not differ significantly between the two groups. A similar double-blind placebo-controlled study was performed by Van Gossum *et al*[143], who randomized 70 patients who had underwent elective ileocecal resection for CD to daily treatment with either *Lactobacillus johnsonii* LA1 10^{10} CFU ($n = 34$) or placebo ($n = 36$) for 12 wk. The primary objective of this study was to assess the effect of probiotics on the endoscopic recurrence rate at 12 wk. Clinical relapse rate was 15% in the probiotic group and 13.5% in the placebo group ($P = 0.79$). The mean endoscopic score at 3 mo was not significantly different between the two groups ($P = 0.48$), nor was the percentage of patients with severe endoscopic recurrence ($P = 0.33$). According to the results of these studies, it seems that, like LGG, *Lactobacillus johnsonii* LA1 has no effect on remission in CD.

With the intention of preventing postoperative recurrence of CD, another two double-blind placebo-controlled trials were performed. Chermesh *et al*[144] investigated the use of a synbiotic cocktail of 4 probiotics and 4 prebiotics (Synbiotic 2000), and Madsen *et al*[145] used multispecies probiotic VSL#3, which proved to be efficient in the therapy of ulcerative colitis and pouchitis.

In the 2006 Cochrane Collaboration review regarding probiotics for maintenance of remission in CD by Rolfe *et al*[146], the authors identified 7 eligible studies. They found no statistically significant benefits of *E. coli* Nissle for reducing the risk of relapse compared to placebo, or for *Lactobacillus rhamnosus* GG after surgical or medically-induced remission. There was no statistically significant benefit of probiotics for reducing the risk of relapse compared to medical maintenance therapy employing aminosalicylates or azathioprine. Moreover, they found more adverse events in *Lactobacillus* GG treated patients. However, a small study using *Saccharomyces boulardii* demonstrated a difference in favor of its use combined with medical maintenance therapy in comparison with standard medical therapy alone, although the difference was

not statistically significant. They concluded that there is no evidence to suggest the use of probiotics for the maintenance of remission in CD. In the second Cochrane Collaboration review analyzing different interventions for the prevention of post-operative recurrence of Crohn's disease, the authors came to the same conclusion that probiotics were not superior to placebo[147].

Similarly, a meta-analysis performed by Rahimi *et al*[148] also failed to demonstrate the efficacy of probiotics in maintaining remission and preventing clinical and endoscopical recurrence in CD. Moreover, in a meta-analysis performed by Shen *et al*[149], researchers came to the conclusion that not only were *Lactobacilli* inefficacious, but also that administration of *Lactobacillus* GG might increase the relapse rate.

PREBIOTICS AND IBD

Compared to probiotics, there is considerably less clinical evidence regarding the use of prebiotics for IBD therapy.

In an early trial by Hallert *et al*[150], the ingestion efficiency of *Psyllium* (*Plantago ovata*, ispaghula husk) for 4 mo compared to placebo was studied for relieving gastrointestinal symptoms in patients with UC in remission. Regarding the symptom's score, ispaghula was consistently superior to placebo ($P < 0.001$) and was associated with a significantly higher rate of improvement (69% *vs* 24%; $P < 0.001$). Based on these results, the authors suggested that ispaghula could be helpful in the management of gastrointestinal symptoms in UC.

A Spanish group performed a multicenter open-label, randomized clinical trial to assess the efficacy and safety of *Plantago ovata* seeds as compared with mesalamine in maintaining remission in UC[151]. A total of 105 patients with UC in remission were randomized into three groups treated with *Plantago ovata* (10 g twice daily), mesalamine (500 mg twice daily), or *Plantago ovata* plus mesalamine at the same doses for 12 mo. Three patients, all from the *Plantago ovata* group, were withdrawn because of adverse events (i.e., constipation and/or flatulence). After 12 mo, the treatment failure rate was 40% in the *Plantago ovata* group, 35% in the mesalamine group, and 30% in the *Plantago ovata* plus mesalamine group. The probability of continued remission was similar ($P = 0.67$). A significant increase in fecal butyrate levels was observed in the groups using *Plantago ovata* ($P = 0.018$). The authors concluded that *Plantago ovata* seeds might be as effective as mesalamine for maintenance therapy in UC patients in remission. Furthermore, Casellas *et al*[152] conducted a prospective, randomized, placebo-controlled pilot trial comparing the effect of oligofructose-enriched inulin 12 g/d ($n = 10$) and maltodextrin used as placebo ($n = 9$) for 2 wk in patients with mild to moderately active UC. Concomitant treatment with mesalazine (3 g/d) was allowed. A significant reduction of fecal calprotectin, a marker of intestinal inflammation, was observed in the group receiving oligofructose-enriched inulin (day 0: 4377 +/- 659 μg/g; day 7: 1033 +/- 393 μg/g, $P < 0.05$) but not in the placebo group (day 0: 5834 +/- 1563 μg/g; day 7: 4084 +/- 1395 μg/g, n.s.).

Hafer *et al*[153] investigated the clinical and histological efficacy of lactulose in patients with both UC and CD. In a pilot study, 14 UC and 17 CD patients, most of whom were in a clinically active state, were randomized either to receive 10 g lactulose daily or placebo, adjuvant to standard therapy for 4 mo. No significant improvement of clinical activity index, endoscopic score, or immunohistochemical parameters was observed in CD or UC patients receiving lactulose in comparison to the control group.

Several clinical trials were performed in Japan using germinated barley foodstuff (GBF), which mainly consists of dietary fiber and glutamine-rich protein, for the therapy of UC. Kanauchi *et al*[154] investigated the efficacy of long-term administration of GBF in the treatment of active UC in a multi-center open trial. Twenty-one patients with mild to moderate UC received 20-30 g of GBF while baseline treatment with 5-aminosalicylates and/or steroids was continued. After 24 wk of treatment, the GBF group showed a significant decrease in clinical activity index compared with the control group ($P < 0.05$). No side effects related to GBF were observed. The same group published results of another study in which GBS was used for maintenance therapy in UC[155]. Patients were randomized into two groups: GBF 20 mg/d ($n = 22$) and control ($n = 37$). Response to treatment was assessed by monitoring the clinical activity index (CAI) and endoscopic score. Significantly better CAI values and a significantly lower recurrence rate were observed in the GBF group at 3, 6, and 12 mo compared with the controls. No side effects related to GBF were observed. According to the results of both studies, GBF could reduce the clinical activity of active UC, and appeared to be effective as a maintenance therapy in patients with UC.

Moreover, a small open-label trial was performed by Lindsay *et al*[156] in which they treated 10 patients with active ileocolonic Crohn's disease with 15 g of FOS for three weeks. FOS induced a significant reduction in the disease activity index from 9.8 ± 3.1 to 6.9 ± 3.4 ($P < 0.01$). They also observed a significant increase in fecal *Bifidobacteria* concentration, in the percentage of IL-10 positive, and TLR2 and TLR4 expressing dendritic cells in mucosal biopsies.

In contrast to previous findings, the results from a randomized double-blind placebo-controlled trial performed by Benjamin *et al*[157] did not confirm the efficacy of FOS for therapy of active CD. In this study patients were randomized to 15 g/d FOS ($n = 54$) or placebo ($n = 49$) for 4 wk. More patients receiving FOS (26% *vs* 8%; $P = 0.018$) withdrew before the 4-wk end point and there was no significant difference in the number of patients achieving a clinical response between the FOS and placebo groups (22% *vs* 39%; $P = 0.067$).

Considering all the above facts regarding the use of prebiotics, there is very little evidence to support their use in IBD therapy. However, supplementation with germinated barley foodstuff, *Psyllium* (*Plantago ovata*, ispaghula

husk), or oligofructose-enriched inulin might provide some benefit in patients with active UC or UC in remission, but more high-quality clinical studies are needed to confirm their effectiveness.

CONCLUSION

Probiotics and prebiotics definitely have great potential for future therapeutic approaches in inflammatory bowel disease. However, further research is required to identify specific probiotic strains or their combinations and prebiotic substances that will be most efficient for therapies of different types and stages of activity of intestinal inflammation.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Clinical characteristics and treatment of inflammatory bowel disease: A comparison of Eastern and Western perspectives

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Abstract

Inflammatory bowel disease (IBD) is a chronic, relapsing intestinal inflammatory disorder with unidentified causes. Both environmental factors and genetic aspects are believed to be crucial to the pathogenesis of IBD. The incidence and prevalence of IBD have recently been increasing throughout Asia, presumably secondary to environmental changes. This increasing trend in IBD epidemiology necessitates specific health care planning and education in Asia. To this end, we must gain a precise understanding of the distinctive clinical and therapeutic characteristics of Asian patients with IBD. The phenotypes of IBD reportedly differ considerably between Asians and Caucasians. Thus, use of the same management strategies for these different populations may not be appropriate. Moreover, investigation of the Asian-specific clinical aspects of IBD offers the possibility of identifying causative factors in the pathogenesis of IBD in this geographical area. Accordingly, this review summarizes current knowledge of the phenotypic manifestations and management practices of patients with IBD, with a special focus on a comparison of Eastern and Western perspectives.

Key words: Crohn's disease; Ulcerative colitis; Inflammatory bowel disease; Clinical characteristics; Treatment; Asia

Core tip: Over the past two decades, the incidence and prevalence of inflammatory bowel disease (IBD) have changed with a trend toward increasing across Asia, especially East Asia. This increasing trend in IBD epidemiology necessitates specific health care planning and education in Asia. To this end, we must gain a precise understanding of the distinctive clinical and therapeutic characteristics of Asian patients with IBD, compared to Caucasians patients with IBD. Accordingly, this review summarizes current knowledge of the phenotypic manifestations and management practices of patients with IBD, with a special focus on a comparison of Eastern and Western perspectives.

Park SJ, Kim WH, Cheon JH. Clinical characteristics and treatment of inflammatory bowel disease: A comparison of Eastern and Western perspectives. *World J Gastroenterol* 2014; 20(33): 11525-11537 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i33/11525.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i33.11525

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic, idiopathic inflammatory disorder of the gastrointestinal tract without identifiable causes. It comprises primarily Crohn's disease (CD) and ulcerative colitis (UC). IBD was traditionally regarded as being prevalent in mainly Western countries. Over the past two decades, however, the incidence pat-

Table 1 Clinical characteristics of inflammatory bowel disease in Asian and Western countries

Differences	Asian characteristics
Peak age at disease onset	Smaller second peak
Sex distribution of CD	Male predominance
Cigarette smoking in CD	Lower prevalence
CD distribution and behavior	Ileocolonic predominance Similar behavior
UC distribution and behavior	Similar distribution Milder disease course
Familial aggregation in IBD	Lower prevalence
Extraintestinal disease	Lower prevalence
Medical treatments	Lower use of thiopurine and anti-TNF therapy
Surgical treatments	Comparable in CD Lower in UC
UC-associated CRC	Comparable cumulative risk

CD: Crohn's disease; UC: Ulcerative colitis; anti-TNF therapy: anti-Tumor necrosis factor therapy; CRC: Colorectal cancer.

tern of IBD has changed. The incidence in the West has remained relatively stable, while that in Asia has increased markedly[1]. This recent change in the incidence and prevalence of IBD is attributed to environmental changes.

The growing incidence of IBD in Asia has important implications for those who formulate health care policy plans; these individuals should provide specific health care planning, services, and education while balancing the health needs and social burdens of a given country. However, it also has important implications for clinicians and researchers. According to the published literature, the IBD phenotypes differ considerably between Asians and Caucasians. Nevertheless, most strategies for the prevention and treatment of IBD flare-up in Asia have followed Western guidelines. Gradual accumulation of data on IBD in Asians will facilitate the formulation of Asian-specific practice guidelines; however, direct comparisons of the clinical characteristics of IBD in Asian and Western countries are few. Formulation of appropriate strategies for Asian patients with IBD must begin with an accurate understanding of the different clinical characteristics of the two populations. These include clinical manifestations and therapeutic aspects, as well as epidemiology. To this end, this review summarizes and compares current knowledge of the phenotypic manifestations and management of patients with IBD in Eastern and Western countries. Furthermore, investigation of the Asian-specific clinical aspects of IBD offers the possibility of identifying causative factors in the pathogenesis of IBD, which will also be covered in this review.

DIFFERENCES IN CD BETWEEN THE EAST AND WEST

Clinical characteristics of CD

Peak age of disease onset: Early studies from Western countries reported that CD was characteristically associated with a bimodal age distribution pattern, peaking at the ages of 20 to 39 years and showing a second smaller peak at the ages of 50 to 79 years[2-4]. Various peak ages of CD onset have been reported in Asia. A Korean study reported a smaller second peak in the incidence of CD[5]. There was also a trend toward a second peak in the Hong Kong population[6]. A recent prospective, population-based study from the Asia-Pacific region also indicated a smaller second peak in the incidence of CD, albeit at a younger age (40-44 years) than that in Western countries[7]. However, this bimodal presentation has not been uniformly identified in other Asian studies[8,9]. The reason for the bimodal distribution remains unclear, but some environmental factors are known to be associated with this phenomenon. One hypothesis is that it may be due to certain age-specific environmental factors, such as passive smoking in childhood and active smoking in adulthood. It may also be caused by the different sensitivities of different age groups to certain infectious factors[10]. We speculate that Asian people with genetic susceptibilities could develop CD at the early peak ages of 20-39 years; however, the later second peak might be smaller than that in the West because environmental factors are less prevalent in Asia. Accordingly, it is expected that the second peak in the incidence of CD in Asia will increase as the region becomes increasingly westernized over time (Table 1).

Male predominance in Asians

European and North American studies have consistently revealed that the incidence of CD in females is equal to or greater than that in males[2,4,11,12]. In striking contrast, a male predominance in Asian patients with CD has been reported. In all recent studies from Korea, China, and Japan, the male-to-female ratios ranged from 1.67:1 to 2.9:1[9,13,14]. A recent prospective, population-based study from the Asia-Pacific region also demonstrated a male predominance[7]. Interestingly, a recent European study indicated a slight male predominance (60%) in adult Eastern European patients with CD[15]. Smoking, vaccination, or other factors (*e.g.*, geographic, ethnic, and social) might account for this sex difference[10]. Some Western researchers have suggested that the smoking rates, exposure of social life, or Westernized lifestyles were more prevalent in males than in females in Asia and that males might have more opportunities to receive medical services, including endoscopy, than females in Asia. However, if male predominance is in fact present in Asia, it is possible that changes in susceptible genes in sex chromosomes or changes in sex hormones might be involved in the pathogenesis of CD.

Cigarette smoking

Smoking represents one of the most consistently observed environmental risk factors for CD. Studies in Western countries have shown that smoking is a strong risk factor for the development of CD, but that it protects against the development of UC[16-20].

Based on recent prospective, population-based cohort

studies, the proportion of current or ex-smokers among Asian patients with CD (11.8%-28.0%)[7,21] is substantially lower than that among Western (57%) and Eastern European patients (62%)[15]. A study comparing patients between Melbourne and Hong Kong also reported that fewer patients were current or ex-smokers in Hong Kong (8%) than in Melbourne (50%)[22].

Western patients with CD who smoke have a worse disease course and are more likely to relapse after medically and surgically induced remission[23-25]. In terms of flare-up rates and therapeutic needs, disease severity is similar in patients who have never smoked and those who have stopped smoking, and both have a better course than continuing smokers[23]. Progression to stricturing or penetrating disease and subsequent surgery rates are reduced by smoking cessation[26]. However, smoking may not have the same effect in CD in different ethnic groups or geographic regions as in Western populations. For example, studies among Israeli Jews showed that smoking is not associated with the risk of CD[27-29]. More studies on Asian patients with CD are warranted to determine the impact of smoking on the development and progression of disease and its association with the disease phenotype in this population. Our hypothesis is that the lower rate of cigarette smoking among Asians may be one of the factors related to their better prognosis, as indicated by the lower rate of surgery in Asian than in Western patients with CD.

Familial aggregation

Previous studies have suggested that patients with IBD have less family clustering in East Asia. Studies from Asia have collectively reported that familial aggregation rates range from 0.0% to 3.0%[6,30-35], which is clearly lower than that in the Western population (13.4%)[36]. However, familial aggregation rates appear to be higher in West Asia, ranging from 12.9% to 19.0%[1,37]. Recent studies from Korea have suggested that the familial aggregation rate of IBD may increase with time, in parallel with the increase in the prevalence of IBD within the country[1,5,31]. Data on the familial clustering rates in other Asian countries must be validated by a longitudinal prospective cohort study. Moreover, it should be tested whether patients with family history of IBD would have a worse disease outcome than those without. Our own study showed no difference in clinical characteristics and outcomes between them[38]. We speculate that both environmental and genetic factors contribute to the low familial aggregation rates among Asian patients with CD. It might be valuable to elucidate the Asian-specific pathogenesis and establish prevention methods by investigating Asia-specific lifestyles or environmental factors, including breast feeding, tonsillectomy, childhood vaccination, infectious disease, or dietary intake of fiber and sugar.

Disease location

In the West, CD has been found to occur in the ileum, colon, and both the ileum and colon in equal proportions of patients[15,39]. However, a number of studies from the West have reported isolated colonic disease to be the most common type of CD[11,40,41]. A study comparing patients in China and the US also reported that American white patients have more colorectal involvement[32]. However, ileocolonic disease appears to be the most common type of CD in Asia[5,7,21,33,34,42,43]. CD confined to the small bowel is also common in Asia. Western guidelines suggest a follow-up colonoscopy to assess mucosal healing or recurrence after biologic therapy or surgery. However, routine ileocolonoscopy for therapeutic monitoring or after surgery might be less useful in Asian patients. Instead, radiological evaluation might play a role in such cases because isolated ileal or ileocolonic disease is not entirely visible by ileocolonoscopy.

Upper gastrointestinal tract involvement, which is significantly associated with disease prognosis such as changes in behavior[44] or the need for early surgery or hospitalization[45], has been rarely reported in Asia. This might be due to the lack of consensus regarding the performance of routine gastroduodenoscopy in patients with newly diagnosed CD in real practice. A study from China found that 23.5% of patients had upper gastrointestinal tract involvement at the time of diagnosis[14], but other studies[3,7,11,46,47] from both the Asia-Pacific region and the West have reported lower proportions. In a recent Korean study, jejunal involvement was observed in 14.1% of patients with CD at the time of diagnosis[48]. This markedly different result compared to China might be an overestimation. Thus, further larger studies are needed to determine the proportion of upper gastrointestinal involvement in patients with CD in Asia.

Disease behavior and course

Studies from the West have shown that the rates of inflammation, stricture, penetration, and perianal fistulas in CD at the time of diagnosis are 62%-81%, 5%-27%, 8%-14%, and 10%-27%, respectively[11,44,47,49,50]. Similarly, Asian studies have reported inflammatory disease in 40% to 69%, stricturing disease in 20%-29%, and penetrating disease in 10%-31% of patients with CD[13,21,33,34,45,51,52]. Interestingly, several studies documenting the disease behavior at the time of diagnosis have reported higher proportions of perianal fistula at the time of diagnosis in Hong Kong (33.3%)[33], Korea (36.7%)[34], and China (58.8%)[14] than in the West. Perianal fistula is known to be a poor prognostic factor in patients with CD[44,47]. In general, however, the prognosis of Asian patients with CD patients is reportedly better than that of Western patients with CD. Whether the presence of perianal fistula is actually a poor prognostic factor of CD remains to be determined and warrants further study. Actually, perianal fistula is described as independent from the penetrating type in the Montreal classification.

The evolution from inflammatory behavior to a more complicated disease behavior (stricturing or penetrating) is well demonstrated in the Western literature[46,47,53], and has also been shown in Asian studies from Hong Kong[33]

and Korea[54].

Based on long-term follow-up studies from Japan, it appears that Japanese patients with CD have a long-term prognosis similar to that of their Western counterparts regarding cumulative operation rates[43]. However, compared with Western patients with CD[55], a recent study from Korea showed a better prognosis with respect to the incidence of surgery[13,34]. This can be partly explained by the conservative attitude regarding bowel surgery among Korean physicians and patients or the smaller numbers of patients with a severe phenotype because of the short history of CD in the Asian region, including Korea.

Extraintestinal manifestations

Extraintestinal manifestations (EIM) have been reported in Asian patients with CD with widely variable rates: 19.0%-58.8% in China[14,52,56] and 25.0% in Hong Kong[6]. Among EIM, a high rate of ankylosing spondylitis (9%) among patients with CD has been reported[6]. A study comparing patients between the US and China reported that American white patients developed more EIM (40% *vs* 20%; OR = 2.63; $P = 0.013$)[32], especially chronic arthralgia (32% *vs* 4%; OR = 13.07; $P < 0.001$). However, it is difficult to directly compare the prevalence of EIM between Asian and Western patients with CD because most Asian reports were hospital-based (not population-based) retrospective studies or prospective cohort studies involving small numbers of patients ($n = 17$, 58.8%)[14]. Moreover, they were performed under different diagnostic criteria for EIM. Generally, the prevalence of EIM in Asia is accepted to be similar or slightly lower than that in the West (19%-25% *vs* 21%-41%, respectively)[57-60]. Less frequent EIM may be associated with the relatively better prognosis among Asian patients than in Western patients because EIM, especially ankylosing spondylitis, is known to be associated with a poorer prognosis in patients with IBD.

TREATMENT OF CD: COMPARISN BETWEEN THE EAST AND WEST

Medical treatments

Conventional therapy: The use of corticosteroids for CD is variable in studies from Asia. In one study, Asian specialists used corticosteroids as the first-line treatment in 50% of patients with mild CD and 84% of patients with moderate CD[61]. Moreover, they used corticosteroids as a maintenance therapy in approximately 25% of patients with CD[61], which might appear to be inappropriately high because corticosteroids have more side effects than placebo or low-dose 5-aminosalicylic acids (5-ASA)[62]. In a recent prospective European study, 54%-55% of patients with CD received corticosteroid therapy as an initial treatment during the first 3 mo of disease in Eastern and Western European centers, but did not during the maintenance phase[15].

Among patients with CD, the overall response rates to corticosteroids in Asia were similar to or better than those in the West. The short-term response rates at 1 mo were > 80% in both Asia and the West (Figure 1A), and 56.6% and 32.0% of patients, respectively, were corticosteroid-free without the need for surgery at 1 year (Figure 1B)[63,64].

The use of thiopurines in Asia also varies among countries. A Korean single-center study reported that thiopurines were used in 63% of patients with CD[34]. A separate Korean study showed that 42% of patients received thiopurines and that the cumulative thiopurine requirement was 9.1% at 1 year, 32.2% at 5 years, and 51.6% at 10 years[65]. Another cross-sectional study from Hong Kong noted that thiopurines were used in 63.6% of patients[22]. The authors of this study stated that the use of thiopurines was significantly less frequent in Hong Kong patients than in Melbourne patients (63.6% *vs* 82.1%, respectively, $P < 0.001$). A single-center review from East China found that 61 of 227 (26.9%) patients had indications for immunomodulator use. However, such agents were prescribed to only 34.0% of the patients, and of these 34.0%, 38.0% received a subtherapeutic dose with no attempt to increase the dose[66]. A recent prospective, population-based study from the Asia-Pacific region reported that only 35% of patients with CD received immunomodulator therapy[7]. Taken together, these results indicate that Asian physicians have a tendency to inappropriately use large amounts of corticosteroids and insufficient amounts of thiopurines. It is important to educate physicians regarding these medications.

There appears to be a higher rate of adverse events, particularly leukopenia, in Asians than in Caucasians when taking thiopurines. Up to 40%-56% of Asian patients may reach the criterion for leucopenia, namely a white blood cell count of < 4000/mm^3[67,68]. The cumulative incidence of myelotoxicity in the Western population was reported to be about 7%[69]. In Asia, however, thiopurine methyltransferase genotyping itself may not be as helpful in identifying patients who are expected to develop myelotoxicity as in Western countries[70], and the metabolites of thiopurines are not widely measured in real practice in many Asian countries. In Korea, therefore, physicians usually start a thiopurine at a smaller dose (*e.g.,* 25 or 50 mg of azathioprine) and gradually increase the dose with regular evaluations of the white blood cell count instead of determining the metabolite concentration. Alternatively, they may maintain lower doses of thiopurines than recommended in the Western guidelines[71].

Biologics: In a recent prospective, population-based cohort study from Europe, the rates of biologics use as an initial treatment during the first 3 mo of disease were reportedly 7% and 2% in Western and Eastern European centers, respectively[15]. This approach was interpreted as top-down therapy or rapid accelerating therapy. However, the economic burden or the reimbursement system can be obstacles to the use of biologics in the very early phase after diagnosis of CD in many Asian countries. An

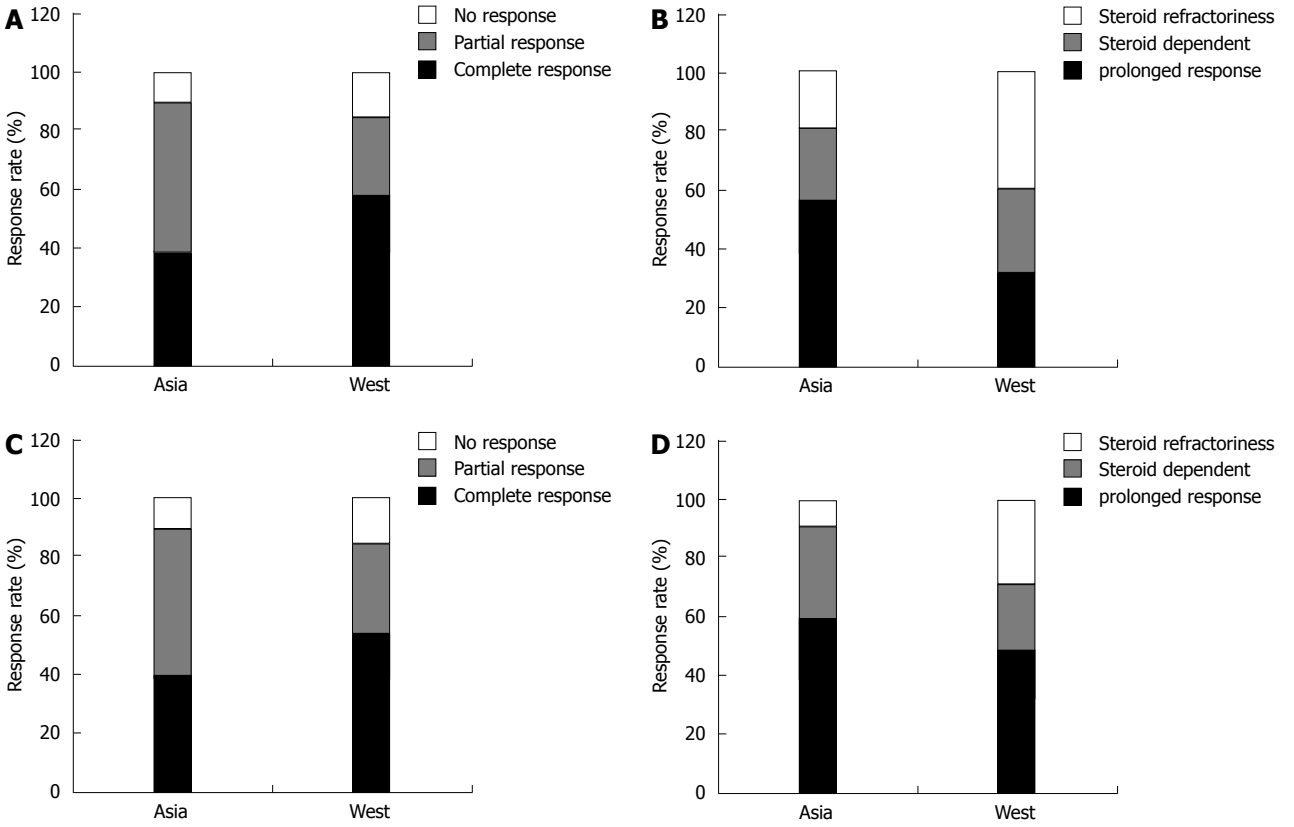

Figure 1 Comparison of the response to corticosteroids between Asia and the West[63,64,112]. A: Crohn's disease, 1 mo; B: Crohn's disease, 1 year; C: Ulcerative colitis, 1 mo; D: Ulcerative colitis, 1 year.

Asian survey of IBD management practices in different countries found that no IBD specialists would consider anti-tumor necrosis factor (anti-TNF) agents as the first choice for the treatment of CD. Moreover, only 20% considered anti-TNF agents as the second choice[61]. In a cross-sectional study comparing the management of CD between Melbourne and Hong Kong, a significantly higher number of patients in Melbourne had been on anti-TNF agents than in Hong Kong (40% *vs* 11%)[22]. A retrospective study from Korea reported that 8.6% of patients with CD used infliximab[34].

The response rates to infliximab in Asia are similar to or higher than those in the West. Although it is difficult to directly compare the results of responses to infliximab in Asia and the West because of their different designs, the response rates at 2 wk after beginning induction therapy were 72% and 62% in Korea (unpublished retrospective data) and the West[72], respectively. Moreover, the response rates at 30 and 54 wk after beginning maintenance therapy were 91.7% *vs* 50.0% and 74.7% *vs* 39.0% in Korea (unpublished retrospective data) and the West[72], respectively. A similar pattern was reported in patients with fistulizing CD between Korea and Western countries. In Asia, anti-TNF agents are used less frequently because of the limited, strict indications under insurance coverage rules, and because of the social economic burden. Moreover, many Asian physicians have not accepted the latest treatment trends, such as rapid accelerated step-up or top-down therapeutic approaches. Again, it is necessary to educate Asian physicians regarding the adequate use of the latest medical treatments for IBD.

Complementary and alternative medicines: There are diverse rates of use of complementary and alternative medicines (CAMs) across Asia. One study from China reported that 90% of patients used concomitant traditional Chinese medications[73]. Moreover, various proportions of Western patients with IBD use CAM, ranging from 23% to 49% in recent studies[74-77]. In seven randomized controlled trials of patients with CD, *Artemisia absinthium* (wormwood) and *Tripterygium wilfordii* were superior to placebo in terms of inducing remission and preventing clinical recurrence of postoperative CD, respectively[78]. In two systematic reviews, omega-3 fatty acids did not appear to be effective for the maintenance of CD remission[79,80]. Effective anti-inflammatory moieties have yet to be defined.

Another problem is the indiscriminate use of various CAMs without sufficient evidence in patients with IBD. More studies are needed to determine the efficacy and safety of CAM in such patients. In addition, the development of new CAMs from natural products would be helpful in situations in which few drugs are available for patients with IBD.

Surgical treatments

The cumulative operation rates for CD in Japanese studies are comparable to those of Western cohorts, ranging

from 25.9% to 44.4% at 5 years and 46.3% to 80.1% at 10 years, respectively[43,81]. The surgical resection rate for patients with CD in Hong Kong (29% at 10 years)[6] is similar to that in a population-based study from Norway, which reported a 10-year cumulative surgical rate of 37.9%[55]. In a cross-sectional study, there was also no significant difference in the proportion of patients with CD who underwent surgery in Melbourne compared with in Hong Kong (55.1% *vs* 46.0%, respectively, $P = 0.065$)[22]. Similarly, the cumulative probability of intestinal resection in Korea was reported to be 15.5% after 1 year, 25.0% after 5 years, and 32.8% after 10-15 years. Asian patients with CD are currently considered to have a similar or slightly lower rate of surgery than that among their Western counterparts[34]. The lack of long-term follow-up studies in Asia makes it difficult to draw a concrete conclusion regarding the cumulative surgical resection rates between Asia and the West.

DIFFERENCES IN UC BETWEEN THE EAST AND WEST

Clinical characteristics of UC

Age and sex: The median age at the time of diagnosis of UC among patients in Asia is similar to or slightly older than that among patients in the West (35-44 years in Asia[5,9,51,82-85] and 30-40 years in the West[39,86,87]). In a cross-sectional study, patients with UC in Hong Kong were diagnosed at an older age than Caucasians in Melbourne (median age, 38 years *vs* 30 years, respectively). The authors suggested that this may be partly explained by a delay in diagnosis in Hong Kong[22]. Another possible explanation would be a weaker influence of genetic factors in Asian patients, which delays disease occurrence (Table 1).

The majority of studies from the West have shown an equal sex distribution for UC, although some reported a male predominance[86,88]. A growing number of studies in Asia have shown an equal sex distribution[5,84,85,87,89] or slight male predominance[9,35,52,82,90]. Collectively, the age and sex distributions of patients with UC are not largely different between the East and West.

Family history: Studies in Asia have reported a family history in 0.0%-3.4% of patients with UC[35,82,85]. This figure is lower than the 10%-25% reported in Western countries[37]. A recent population-based cohort study conducted in the Asia-Pacific area showed a family history in 3% of patients in Asia and in 17% of patients in Australia ($P < 0.001$)[7]. Interestingly, in Korea, an increase in the prevalence of a positive family history from 1.3% in 2001 to 2.7% in 2005[5] paralleled the increased incidence of IBD. This suggests that the low prevalence of a family history may be a reflection of the low population prevalence and will probably change with time.

In terms of genetic associations, a previous Japanese genome-wide association study (GWAS) and a recent Korean GWAS showed considerable overlap of genetic associations for UC between Asia and the West. Despite the overlap of genetic associations of Asian and Western patients with UC, as can be seen by the lower proportion of patients with a family history of UC in Asia compared with the West, we realize that UC is one of the principal forms of IBD with complex manifestations, and genetic factors that account for only a portion of the overall disease development. This indicates a need to better explore gene-environment interactions or Asia-specific environmental factors of etiological importance in the development of IBD.

Disease extent: In UC, the extent of disease is classified into three types: proctitis, left-sided colitis, and extensive colitis. In Western population-based studies, these three types comprise 30%-60% of cases, 16%-40% of cases, and 18%-35% of cases, respectively[91-93]. In Asian population-based studies, they comprise 25.0%-43.7%, 31.0%-31.4%, and 24.9%-39.0%, respectively[5,42]. Most hospital-based studies in Asia have shown a trend toward a lower proportion of proctitis (8.5%-38.4%), higher proportion of left-sided colitis (29.7%-70.2%), and similar proportion of extensive colitis (21.3%-42.4%) compared with population-based studies[35,52,84,85,94], indicating that more severe cases were recruited into the hospital-based studies.

In terms of age and disease extent, several studies have reported that the extent of UC at diagnosis differs significantly according to age at diagnosis[83,95]. In one Korean study, proctitis was more common in elderly patients (28.9% in the young group *vs* 33.8% in the elderly group) and extensive colitis was more common in younger patients (35.1% in the young group *vs* 22.5% in the elderly group), suggesting a poorer clinical outcome in younger patients ($P < 0.05$)[83].

In a recent comparative epidemiological study of IBD across Asia and the Pacific, the extent of UC was classified as proctitis in 37%, left-sided colitis in 32%, and extensive colitis in 31%. These results are not significantly different from those in a study from Australia, which classified UC as proctitis in 32%, left-sided colitis in 27%, and extensive colitis in 41% (all $P > 0.05$)[7]. A recent population-based cohort study from the West and East Europe showed that the ratios of disease extent for UC from Western and Eastern European centers were proctitis in 20% and 22%, left-sided colitis in 41% and 46%, and extensive colitis in 38% and 32%, respectively[15]. Collectively, a slightly higher proportion of extensive colitis is observed in Western countries than in Asia, suggesting a more favorable prognosis of UC in Asia.

Disease course: Although definitions of clinical relapse and measurements of disease severity vary among studies, most suggest that Asian patients with UC have a milder disease course than do patients from Western countries[82,90,96]. In Asian studies, most patients with UC had a chronic relapsing disease course rather than continuous active disease[84,97], similar to Western data[98]. In a Malay-

sian study, the rate of maintaining remission was reported as 64.3% of patients with UC at 10 years after diagnosis, the rate of a chronic relapsing disease course as 25%, and the rate of chronic persistent disease with low or high activity as 3% after 10 years[97]. In a Norwegian study, the rate of maintaining remission was reported to be 55% of patients with UC at 10 years after diagnosis, which are slightly lower than the Malaysian data[98], and the rate of a chronic relapsing disease course to be 37%. In a Korean study, the cumulative relapse rate at 10 years after diagnosis (88.4%) was similar to that of a Western study (83%). However, the cumulative probabilities of colectomy (2.0% after 1 year, 2.8% after 3 years, and 3.3% after 5-15 years)[96] were lower than those in a Western study (3.5% after 1 year, 7.6% after 5 years, and 9.8% after 10 years)[98].

Based on the larger numbers of patients with a remission status, lower numbers of patients with a chronic relapsing or persistent active disease course, and lower cumulative operation rates, Asian patients with UC appear to have a milder disease course than that of Western patients with UC. However, the above-mentioned lower cumulative surgical rates may also be associated with diversity in management strategies or different levels of acceptance of colectomy by physicians and/or patients between Asia and the West in addition to disease severity.

Incidence of colorectal cancer

The overall prevalence of colorectal cancer (CRC) associated with UC is reportedly 3%-5% in the West[99] and 0.0% to 2.2% in Asia[35,52,85,90,96,100-103]. In a previous meta-analysis from the West, the cumulative risk of CRC associated with UC was reportedly 1.6%, 8.3%, and 18.4% at 10, 20, and 30 years, respectively[104]. In Asian studies, the cumulative risk of CRC in patients with UC was reportedly 0.70% to 1.15% at 10 years, 3.56%-7.90% at 20 years, and 14.4%-33.2% at 30 years[100,103], which is comparable with Western cohorts. However, considering the hospital-based design of most Asian studies, the actual corresponding risks might be lower than estimated.

Recently, however, a Western report showed that the risk of CRC decreased from 1979 to 2008 (RR = 1.34 in 1979-1988 to 0.57 in 1999-2008) and that the overall risk of CRC among patients with UC was comparable with that of the general population (RR = 1.07; 95%CI: 0.95-1.21). These findings suggest that a diagnosis of UC no longer seems to increase patients' risk of CRC. However, subgroups of patients with UC, including those diagnosed with UC in childhood or as adolescents, those with a long duration of disease, and those with concomitant primary sclerosing cholangitis, remain at increased risk[105].

Current evidence indicates that the risk of CRC in Asian patients with UC is slightly lower than that in Western patients. There is a chance that the prevalence of CRC will increase with the rising incidence and increasing proportion of patients with a longer follow-up in Asian countries. A very recent single-center study in Korea showed a substantial increase in CRC among patients with long-standing UC, which is comparable to Western data (unpublished data). Long-term prospective follow-up studies are warranted to estimate the actual risk of CRC in Asian patients with UC.

Extraintestinal manifestations

Although there are limited numbers of long-term prospective cohort studies and variations in the definition of EIM in Asian studies, the prevalence of EIM in UC seems to be lower in Asian than in Western countries[58,59,73]. The most commonly involved sites of EIM differ between Asia and the West. The most commonly involved site of EIM is the joints in Asian patients with UC (2.0%-19.5%). Next, eye and skin involvement accounts for 0.0%-4.2% and 0.0%-4.2%, respectively[35,52,82,90,97]. In contrast, eye involvement (iritis/uveitis) in females (3.8%) and primary sclerosing cholangitis (PSC) in males (3.0%) were the most commonly involved sites in a Western population-based study[58].

PSC associated with UC is less prevalent in Asia (0.0%-1.7%)[52,84,96,97,106] than in the West (1.6-7.0%)[59,107]. Because PSC is associated with a risk of CRC in patients with UC, a diagnosis of PSC should not be neglected in real practice despite the low prevalence of PSC in Asia.

TREATMENT OF UC: COMPARISN BETWEEN THE EAST AND WEST

Medical treatments

Conventional therapy: In a recent population-based cohort study in the Asia-Pacific area that compared UC treatments in the first year between Asia and Australia[7], treatment with antibiotics (22% *vs* 14%, $P = 0.15$) and immunomodulators (thiopurine or methotrexate) (18% *vs* 9%, $P = 0.05$) did not differ between Asia and Australia. Mesalazine (79% *vs* 62%, $P = 0.012$) and corticosteroids (62% *vs* 28%, $P < 0.0001$) were more commonly prescribed for IBD at the time of diagnosis in Australia than in Asia. Topical therapy (mesalazine or corticosteroids) for UC was also more frequently prescribed in Australia than in Asia (55% *vs* 28%, $P = 0.04$).

In a recent population-based European study, 69 (44%) patients with left-sided colitis and 19 (23%) with extensive colitis in Western Europe received more frequently receive combination therapy with oral and topical 5-ASA compared with 21 (42%) and 6 (22%), respectively, in Eastern Europe[15]. In this study, 26%-33% of patients with UC received corticosteroid therapy as initial treatment during the first 3 mo of disease in Eastern and Western European centers.

In an Asian survey of management practices for IBD in different countries[61], 83%, 75%, and 61% of respondents preferred 5-ASA, a combination of topical and oral 5-ASA, and corticosteroids, respectively, as induction treatment of mild-to-moderate UC. Almost all respondents agreed that maintenance therapy should be recommended for patients with IBD in remission, with most recommending the use of 5-ASA to maintain remission in UC (91%). However, they also replied that thiopurines and corticosteroids were needed to maintain the remis-

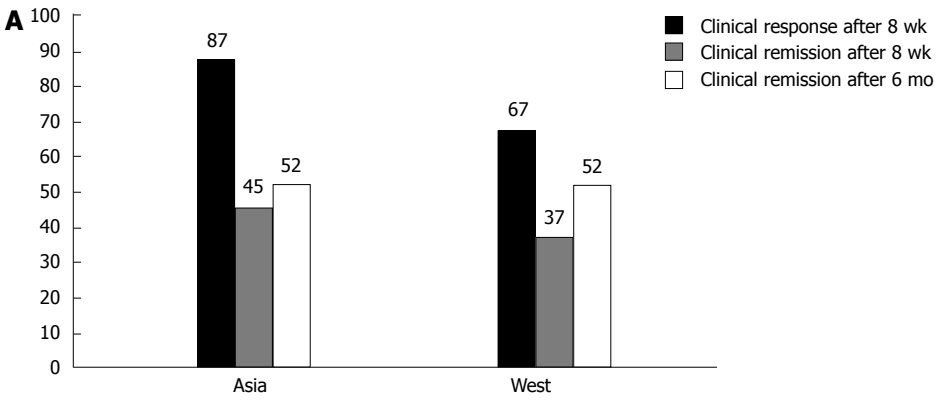

Figure 2 Comparison of the response to infliximab in patients with ulcerative colitis between Asia and the West[113-115].

sion in approximately 30% and 13% of patients with UC, respectively.

In one cross-sectional study, there was less use of corticosteroids (15.3% *vs* 46.5%, $P < 0.001$) and thiopurines (19.7% *vs* 55.3%, $P < 0.001$) for UC in Hong Kong than in Melbourne, which also reflects differences in practice according to region[22]. The authors suggested that Asian physicians prefer to manage UC with less intense medical treatments despite more extensive UC and that they use less thiopurines for maintenance therapy compared with the physicians in Melbourne. Again, it is important to educate Asian physicians in terms of following the adequate use of medical treatments according to the practice guidelines for IBD.

Among the UC patients who receive 5-ASA or sulfasalazine therapy, 49.6%[108] and 72.0% to 75.0%[109,110] experienced disease relapse in Asia and the West, respectively. The cumulative relapse rate was 21.5% after 1 year, 36.5% after 2 years, 46.9% after 3 years, and 59.8% after 5 years during maintenance therapy with 5-ASA/sulfasalazine, and both the disease extent at diagnosis and anemia were major predictive factors for clinical relapse after 5-ASA/sulfasalazine therapy for Korean patients with mild to moderate UC[108].

The overall response rates of Asian patients with UC to corticosteroids are similar to or better than those of patients in the West. Short-term response rates at 1 mo were more than 89.2% and 84.0% in Asia and the West (Figure 1C), and 59.4% and 49.0% showed a prolonged response at 1 year, respectively (Figure 1D)[64,111].

Biologics

A significant difference in the use of anti-TNF inhibitors between Hong Kong and Melbourne has been shown (2/203 *vs* 12/159, $P = 0.001$)[22]. An Asian survey of IBD management practices in different countries found that < 15% of Asian physicians would use anti-TNF therapy in the management of UC[61]. In many countries in Asia, the use of biologic agents is self-financed, making the high cost an obstacle to their wider use. However, a short-term population-based study showed that treatment with biological therapy in the first year after diagnosis (2.0% *vs* 0.0%, $P = 0.21$) did not differ between Asia and Australia[7]. Long-term follow-up studies are needed to show the chronological trends in the use of anti-TNF therapy in Asia. Emerging studies suggest that anti-TNF therapies are effective and safe in Asian patients with UC. The rates of clinical response and remission to infliximab were 87% and 45% in patients with UC at week 8[112], which is slightly higher than the rate of clinical response in the West (69.4%-64.5% at week 8)[113] (Figure 2). Data on long-term efficacy were obtained from 85 of 134 Korean patients who were followed up for more than 6 mo after the first dose of infliximab, and 44 of them (52%) were in remission, which is compatible with UC data in the West[114]. An another recent Korea study reported that 66.3% of patients demonstrated a clinical response at week 8, 32.6% of whom were determined to be in clinical remission[115]; this is similar to the response rates in previous Western reports. Collectively, a similar or slightly higher response rate of infliximab in patients with UC is observed in Asia than in Western countries. In the future, it is expected that other anti-TNF agents, such as adalimumab and golimumab, will be used more widely in addition to infliximab in patients with UC in Asian countries.

Complementary and alternative medicines

In 14 randomized controlled trials of patients with UC, aloe vera gel, *Triticum aestivum* (wheat grass juice), *Andrographis paniculata* extract (HMPL-004), and topical Xilei San were superior to placebo in inducing remission or response, and curcumin was superior to placebo in maintaining remission. *Boswellia serrata* gum resin and *Plantago ovata* seeds were as effective as mesalazine, whereas *Oenothera biennis* (evening primrose oil) was not effective and had relapse rates similar to those of omega-3 fatty acids in the treatment of UC[78]. Larger controlled studies with stricter endpoints and better-defined patient groups are required to obtain more conclusive findings regarding the use of CAM in IBD.

CONCLUSION

Over the past two decades, the incidence and prevalence of IBD have changed with a trend toward increasing

across Asia, especially East Asia. A younger second peak age of disease onset has been shown in Asian populations with CD compared to in Western populations. There is a predominance of male sex and ileocolonic involvement among Asian patients with CD. In patients with UC, the age and sex distribution are not different between Asia and the West. The proportion of current or ex-smokers among Asian patients with CD is lower than that among Western patients. The familial aggregation rates of patients with CD and UC are lower in Asia, but appear to be higher in the West. The disease extent in Asian patients with UC is not significantly different from, and may be slightly less severe than, that in Western populations. Asian patients with UC seem to have a milder disease course than do patients in Western countries. PSC associated with UC is less prevalent in Asia than in the West. Cumulative surgical resection rates in patients with CD do not appear to be different between Asia and the West despite the lack of large-scale, long-term follow-up studies in Asia. Whereas the cumulative surgical resection rates in Asian patients with UC are lower than those in Western patients, the cumulative risk of CRC associated with UC among Asian patients with UC is reportedly comparable with that among Western patients. The use of thiopurine or biologics in patients with IBD remains less frequent in Asia than in the West. There appears to be a higher rate of adverse events, particularly myelotoxicity, in Asians than in Caucasians prescribed thiopurines. The treatment responses for corticosteroids, thiopurines, and biologics of Asian patients with IBD are slightly better than or comparable to those of Western patients.

Several recent prospective, population-based cohort studies were conducted in Asia. Long-term follow-up results from these cohort studies are warranted to help clinicians and researchers further objectively compare the disease prognosis between Asian and Western countries, provide specific health care planning and education, and offer the possibility of identifying causative factors in a population with a rapidly increasing incidence in Asia.

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TOPIC HIGHLIGHT

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

Transanal endoscopic surgery in rectal cancer

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Abstract

Total mesorectal excision (TME) is the standard treatment for rectal cancer, but complications are frequent and rates of morbidity, mortality and genitourinary alterations are high. Transanal endoscopic microsurgery (TEM) allows preservation of the anal sphincters and, *via* its vision system through a rectoscope, allows access to rectal tumors located as far as 20 cm from the anal verge. The capacity of local surgery to cure rectal cancer depends on the risk of lymph node invasion. This means that correct preoperative staging of the rectal tumor is necessary. Currently, local surgery is indicated for rectal adenomas and adenocarcinomas invading the submucosa, but not beyond (T1). Here we describe the standard technique for TEM, the different types of equipment used, and the technical limitations of this approach. TEM to remove rectal adenoma should be performed in the same way as if the lesion were an adenocarcinoma, due to the high percentage of infiltrating adenocarcinomas in these lesions. In spite of the generally good results with T1, some authors have published surprisingly high recurrence rates; this is due to the existence of two types of lesions, tumors with good and poor prognosis, divided according to histological and surgical factors. The standard treatment for rectal adenocarcinoma T2N0M0 is TME without adjuvant therapy. In this type of adenocarcinoma, local surgery obtains the best results when complete pathological response has been achieved with previous chemoradiotherapy. The results with chemoradiotherapy and TEM are encouraging, but the scientific evidence remains limited at present.

Key words: Rectal cancer; Rectal adenocarcinoma; Transanal endoscopic microsurgery; Transanal endoscopic surgery; Colorectal cancer

Core tip: This review describes the indications for local surgery for rectal cancer using transanal endoscopic microsurgery (TEM). Careful selection of patients with T1 adenocarcinomas is required. We describe the promising results obtained in T2 adenocarcinoma with a combination of TEM and preoperative chemoradiotherapy.

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INTRODUCTION

Total mesorectal excision (TME) is the standard treatment for rectal cancer. It achieves locoregional control

of the disease, and the rate of local recurrence is below 5%[1]. TME involves low anterior rectal or coloanal resection, very often combined with a protective ostomy, or abdominoperineal resection (Miles operation) and definitive colostomy. However, complications are frequent; associated morbidity is around 33%, mortality 2%[2], and 20%-30% of patients present genitourinary alterations and sexual dysfunction[3,4].

The capacity of local surgery to cure rectal cancer depends on the degree of lymph node invasion. The risk of possible metastatic lymph nodes has been reported to range between 0% and 12% in T1, between 12% and 28% in T2 and between 36% and 79% in T3[5]. In local surgery, endoanal excision is limited by the height of the lesion with respect to the anal verge; it is difficult to control the resection limits and to perform complete removal of the rectal wall. Local surgery *via* trans-sphincteric exposure as described by Mason[6] has been used to treat lesions in the middle third of the rectum located in the anterior face, but the sectioning of the sphincters raises the morbidity rate. Kraske's trans-sacral rectal excision[7] made it possible to reach the upper third of the rectum, but it has also been abandoned due to its high morbidity and mortality.

Transanal endoscopic microsurgery (TEM) provides a solution to these problems. First described by Buess *et al*[8], this endoscopic procedure allows preservation of the anal sphincters and, through its excellent viewing system, allows access to rectal tumors as far as 20 cm from the anal verge. TEM facilitates the maneuvers of dissection, cutting, coagulation and suturing. Postoperative morbidity rates are below 10%, and no mortality, genitourinary alterations or sexual dysfunction have been reported[9,10].

So what is the place of local surgery using TEM in rectal cancer? In this review, we examine the following aspects of its use: patient selection; surgical technique and types of equipment; risk of adenocarcinoma in rectal adenomas; its indication in T1 rectal adenocarcinomas, and its application in T2 tumors.

SELECTION OF PATIENTS FOR TEM: TREATMENT GROUPS

All possible candidates for TEM must undergo full preoperative staging of the tumor: total colonoscopy with multifocal biopsy, and rigid rectoscopy prior to endorectal ultrasound (EUS), to confirm tumor size, the distance of its lower and upper edge from the anal verge, and location by quadrant (anterior, posterior, right or left lateral). EUS allows staging of the lesion according to Hildebrandt's criteria[11] and pelvic magnetic resonance imaging (MRI) is an important complement, although MRI is not more accurate than EUS, in rectal adenocarcinomas it is needed to confirm the tumor stage and the absence of metastasis to lymph nodes.

If adenocarcinoma is either suspected or has been diagnosed, abdominal and chest computed tomography is performed to rule out distance metastasis and to determine tumor markers carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9. All patients are administered the Wexner sphincter function questionnaire[12], if there are signs of fecal incontinence, anorectal manometry is performed to obtain baseline parameters. We have found that TEM causes manometric alterations but does not affect clinical continence scores[13].

After these complementary examinations, patients are classified into preoperative indication groups from I to IV[10,14]. Group I, with curative intent, includes rectal lesions with biopsy revealing adenoma and staged uT_0, uN_0 by EUS and pelvic MRI. Group II, also with curative intent, includes adenocarcinomas [either well differentiated (G1) or moderately differentiated (G2)], and staged uT_{0-1}, uN_0. Group III, indication by consensus, includes adenocarcinomas [either well differentiated (G1) or moderately differentiated (G2)], with staging uT_2, uN_0[15]. Group IV includes palliative indications regardless of the tumor stage. Therefore, it is patients in groups I and II who are candidates for TEM.

Certain rectal and pelvic pathologies are habitually treated by laparotomy or laparoscopy *via* an abdominal approach. The use of TEM by expert groups allows some of these surgeries to be performed using a less aggressive approach which achieves lower morbidity rates. These indications are termed "atypical", as they do not involve removal of rectal tumors[16,17].

SURGICAL TECHNIQUE

On the day prior to surgery all patients undergo mechanical preparation of the colon and thromboembolism prophylaxis. With the induction of anesthesia, they are administered the standard antibiotic prophylaxis in colorectal surgery.

In the classical technique of TEM[8], correct positioning of the patient on the operating table is vitally important. In TEM the surgeon works with the tumor visible in the lower part of the rectoscope at all times, so the positioning of the patient depends on the location of the rectal tumor. The TEM equipment comprises a 4 cm diameter rectoscope with two different lengths (12 and 20 cm) selected according to the location of the tumor. The pneumorectum is maintained at a constant pressure (10-12 mmHg). The rectal distension created in this way exposes the tumor and the rectal wall. Our group[10] begins the dissection by making a dotted line with the monopolar electric scalpel 10-15 mm from the tumor. We then open the mucosa over the dotted line and begin the full thickness excision of the rectal wall using an ultrasound scalpel (Ultracision, Ethicon, Endo-Surgery, Cincinnati, OH, United States). The defect of the lesion in the rectal wall is sutured to avoid stenosis of the rectal lumen and postoperative bleeding due to feces. The suture is made transversally so as not to compromise the rectal lumen.

Oral diet is initiated on the day after surgery and increased progressively depending on tolerance. Standard analgesia is administered with non-steroid anti-inflamma-

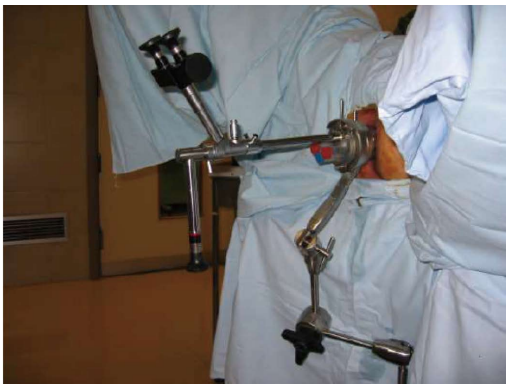

Figure 1 Transanal endoscopic microsurgery equipment.

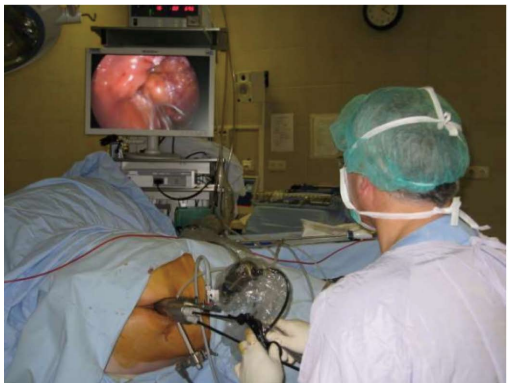

Figure 2 Transanal endoscopic operation equipment.

tory drugs and morphine as rescue medication. The bladder catheter is withdrawn after surgery and the patient is mobilized after eight hours. The patient is discharged from hospital between days 2 and 4 post-surgery.

Different types of transanal endoscopic surgery

As noted above, TEM[8] is an endoscopic procedure with three-dimensional vision (3-D) (Figure 1). Transanal endoscopic operation (TEO) provides two-dimensional vision also through a 4 cm diameter rectoscope of various lengths (7.5, 15 and 22 cm) and on-screen vision. The introduction of a high-definition camera and its application in a panoramic thin-film transistor screen provides an image that is very similar to 3-D. The surgeon is seated in front of the monitor, as in laparoscopy; this makes the learning process easier (Figure 2). Like Nieuwenhuis *et al*[18], we performed a comparative study of our experience with the 2-D system (TEO) and the classical 3-D system (TEM). Our results with regard to surgical difficulty, postoperative morbidity, quality of surgical resections were similar for the two approaches, but the economic cost was lower in the case of TEO[19].

Recently a new transanal endoscopic surgery technique has been introduced termed transanal minimally invasive surgery (TAMIS)[20] or transanal single port microsurgery (TSPM)[21], which uses a single laparoscopic port *via* the anus (Figure 3). We have considerably less experience with TAMIS/TSPM approaches than with TEM

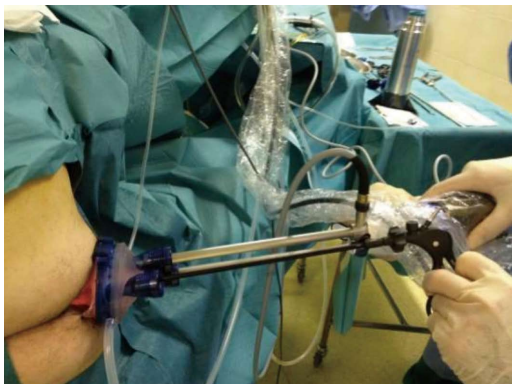

Figure 3 Transanal minimally invasive surgery or transanal single port microsurgery equipment.

or TEO. TAMIS/TSPM requires an assistant to hold and move the camera. From the technical point of view, the introduction of the single port into the anal canal is more complex than in TEM/TEO; a further disadvantage is that the rectoscope cannot be mobilized at the site of the lesion, a maneuver that can be performed with TEM/TEO.

We carried out an economic study comparing TEM/TEO/TAMIS based on the following assumptions: The economic aspects are analysed annually [estimating 50 surgical interventions (SI) per year and a useful life of the non-expendable material of 5 years], divided into variable and fixed costs; The variable annual costs are determined by applying the equation (1): *n* annual surgeries × (surgical time + hospital stay + consumable material). Consumable material: Ultracision scalpel, single port, sutures…; The annual fixed costs are determined by applying the equation (2): non-expendable equipment/time of useful life; The annual cost for each technique is given by the equation (3): annual fixed costs + annual variable costs; the estimated cost of the use of the operating theatre is 10 €/min and the cost of hospitalization in a conventional ward is 220 €/d.

The variable annual costs calculated are [equation (1)]: TEO: 93000 €/year [50 SI × (700 €/SI + 660 €/SI + 500 €/SI)]; TEM: 104500 €/year [50 SI × (830 €/SI + 660 €/SI + 600 €/SI)]; TAMIS: 111000 €/year [50 SI × (760 €/SI + 660 €/SI + 800 €/SI)].

The fixed annual costs calculated are [equation (2)]: TEO: 3000 €/year (15000 €/5 years); TEM: 11000 €/year (55000 €/5 years); TAMIS: 0 €/year.

The total annual costs calculated for each technique are [equation (3)]: TEO: 96000 €/year (93000 €/year + 3000 €/year); TEM: 115500 €/year (104500 €/year + 11000 €/year); TAMIS: 111000 €/year.

Finally, under these assumptions, we obtain the following mean costs: TEO: 1920 €, TEM: 2310 €, TAMIS: 2220 €.

Technical limitations of TEM: Height and morphology

Limitations due to height: The distance of the upper edge of the lesion from the anal verge is of vital

importance. Conventional endoanal excision is limited to lesions at distances of up to 7-8 cm. With TEM, classically the limits were set by the risk of perforation of the peritoneal cavity: it was possible to perform the excision with a low risk of perforation at a distance of up to 18-20 cm when the tumor was located in the posterior quadrant, and up to 15 cm when its location was anterior or lateral. Today, perforation of the peritoneal cavity is not considered a contraindication for TEM[22]. There are no limits in terms of the location of the lesion (*i.e.*, anterior, posterior, or lateral). The limit due to height is determined by the length of the rectoscope, and on occasion by anatomical features: narrow rectosigmoidal junctions with a small rectal ampulla (below 10 cm), or a history of abdominal surgical interventions that immobilize the rectosigmoidal junction and impede the progression of the rectoscope further than 10 cm. The limit for low lesions is the anal verge itself.

Limitations due to morphology: It is possible to excise adenomatous lesions that cover up to three quadrants of the circumference (10-12 cm Ø). In fact all four quadrants can sometimes be reached if the lesions are not particularly wide and if the size does not exceed the height permitted. The problem presented by large lesions is the need to suture the defect, due to the risk of stenosis. If the defect cannot be completely closed, it should be reduced to the maximum - especially the upper part, due to the risk of perforation.

Follow-up protocol for rectal adenocarcinomas after TEM

In accordance with international guidelines, in our treatment group Ⅱ we recommend strict follow-up of these lesions. The follow-up schedule comprises rectosigmoidoscopy-biopsy, EUS and CEA determination every four months during the first and second years; rectosigmoidoscopy-biopsy, EUS and CEA every six months from the third to fifth year; complete colonoscopy, abdominal CT and pelvic MRI annually until the fifth year; and from the fifth year onward, the standard follow-up protocol for colon polyps. The usefulness of EUS after TEM is limited due to the difficulty of interpreting the scar fibrosis, and so it is substituted by pelvic MRI.

HIGH FREQUENCY OF ADENOCARCINOMA IN LARGE RECTAL ADENOMAS

Colorectal adenomatous polyps are considered premalignant lesions with a risk of developing into adenocarcinoma[23]. Early detection and removal are the best means to avoid the appearance of adenocarcinoma[23,24]. In our series and in the study by Absar and Haboubi[25], the rate of invasive adenocarcinomas in adenomatous polyps of the colon was above 18%. Therefore, almost one of every five rectal tumors with a biopsy of adenoma is likely to be an invasive adenocarcinoma. For this reason, piecemeal endoscopic resection or mucosectomy is insufficient treatment[26]. In the case of large rectal adenomas we advocate full-thickness rectal wall resection using TEM, leaving adequate safety margins for correct staging by the pathologist[27]. In our series, half of the infiltrating adenocarcinomas resulting from adenomas were pT1[26]. This means that, with adequate resection and pathological diagnosis, in the absence of factors of poor prognosis, these patients will not require radical rescue surgery[14,28,29].

The factors associated with malignancy in rectal adenomatous tumors have not been clearly established. Among epidemiological variables, it has been postulated that male patients aged under 65 may present a higher risk of adenocarcinoma. Nonetheless, multivariate analyses have not identified age or sex as predictive factors[30,31].

Among morphological factors, an association between lesion size and malignancy risk has often been proposed. Years ago, Muto *et al*[32] reported that with lesion size above 2 cm the risk rises to 53%. Other authors have suggested an association between the size of colorectal adenomas and the risk of adenocarcinoma, but have not been able to demonstrate it[33]. Several studies have also established that villous adenomas present a high risk of malignancy. In our series we have not found differences with respect to tubular and tubulo-villous adenomas; however, the sessile type presents a higher risk of adenocarcinoma than other morphologies[30,31,34]. As for dysplasia, it is natural to assume that lesions with high-grade dysplasia present a higher risk of malignancy[30,33].

If the preoperative study of these lesions includes only rigid rectoscopy and/or colonoscopy with biopsies there is a high risk of understaging, because these techniques do not provide information on the extent of the invasion of the wall and the possible lymph node involvement in the case of infiltrating adenocarcinoma[35]. For this reason, the preoperative study of these lesions should include EUS and pelvic MRI[34]. Preoperative EUS identifies lesions with invasion beyond the submucosa (T1)[11,36], and we regard pelvic MRI as an important complement to endorectal ultrasound, even though it is less effective in discriminating between lesions affecting the submucosa and the muscle layer (T1 and T2), it identifies lesions of stages above T2, and can also detect the presence of lymph nodes in which metastasis is suspected[37].

T1 RECTAL ADENOCARCINOMA: TUMORS WITH GOOD OR POOR PROGNOSIS

The treatment of rectal tumors depends on several factors of prognostic significance: the penetration of the tumor in the thickness of the rectal wall, the involvement of the mesorectal fascia, and the presence of lymph node and distance metastasis[38]. According to the TNM classification, T1 rectal adenocarcinoma presents invasion of the submucosa, but not beyond[39-41].

Local surgery is an alternative to TME for treatment

of T1. In long-term series using classical endoanal resection, local recurrence rates are as high as 29%[9,42,43]. TEM has demonstrated its effectiveness in treating these tumors[44] and achieves initial results for local recurrence below 10%. Recently, however, some alarming figures for local recurrence of T1 with TEM have been published[45], and Doornebosch *et al*[46] also reported a rate of 20.5%. Tytherleigh *et al*[39] offered a possible explanation for these high rates by classifying T1 rectal adenocarcinoma according to good or poor prognosis, which may be related to surgical and pathological factors.

The depth of the submucosal invasion is considered the most important predictor of locoregional lymph node involvement[39,41]. Several methods have been described to assess submucosal invasion. All of them present advantages and disadvantages, and there is no single system based on scientific evidence that can be recommended for all situations. At present the Haggitt classification is proposed for polypoid lesions and the Kikuchi classification for non-polypoid lesions[39,40,47,48].

Haggitt *et al*[48] staged polypoid lesions according to the invasion of the carcinoma, as follows: invading the mucosa (level 0), invading the submucosa but limited to the head of the polyp (level 1), invading the neck (level 2), invading any part of the stalk (level 3), or invading beyond the stalk or base (level 4). Level 4 is associated with a high risk of locoregional lymph node involvement.

Kikuchi *et al*[47] divided the invasion of the submucosa into three levels: Sm1, submucosal invasion that does not extend beyond 200-300 μm from the muscularis mucosae; Sm2, intermediate submucosal invasion; Sm3, submucosal invasion near the surface of the muscularis propria. In this classification, the frequency of locoregional lymph node involvement varies according to the depth of the submucosal invasion: 2% in Sm1 lesions, 8% in Sm2 lesions, and 23% in Sm3 lesions. So, in the absence of other risk factors, Sm3 is sufficient to indicate radical surgery.

The Kikuchi classification can be related to the Haggitt levels: levels 1, 2 and 3 correspond to Kikuchi Sm1, while Haggitt level 4 may be Sm1, Sm2 or Sm3[39].

In addition to the depth of submucosal invasion, other predictors of locoregional lymph node involvement have also been reported in the literature. These include the degree of tumor differentiation, vascular invasion, lymph node invasion, perineural invasion, involvement of the resection margin ($\leqslant$ 1 mm), lymphocyte infiltration, tumor budding (presence of neoplastic cells below the invasive front), demarcation of the submucosal invasive front, and tumor differentiation at the leading edge of the lesion[38-41].

Adequate selection of patients for local surgery on the basis of pathological criteria is essential. In addition, the surgical procedure must comply with a series of standards to ensure its effectiveness: complete excision of the lesion in a single piece (*i.e.*, without fragmentation), complete rectal wall excision, and tumor-free resection margins of at least 1 mm from the lesion[38,39,46,49].

In conclusion, if the histological and surgical characteristics are favorable the risk of local recurrence is below 5%, and the lesion is considered to be a T1 rectal adenocarcinoma with good prognosis[39]. T1 with poor prognosis are lesions with predictors of lymph node involvement and deficient surgery (for example, fragmentation, surgical margins affected, or less than 1 mm from the lesion). In these circumstances the risk of local recurrence can rise to 29%.

LOCAL SURGERY IN T2 RECTAL ADENOCARCINOMA

According to the NCCN-2013[50], the standard treatment of rectal adenocarcinoma T2N0M0 (ADC-T2) is TME without adjuvant therapy. These guidelines no longer propose local surgery associated with adjuvant therapy as an alternative, as they did in 2008[51]. The local surgical approaches for ADC-T2 described in the literature are simple local excision (either *via* endoanal excision or TEM), local surgery with postoperative chemoradiotherapy (CT-RT), and preoperative CT-RT and local excision[14,49,52-55]. As we noted above, radical surgery (TME) reduces quality of life and may lead to death due to causes not directly related to cancer. So it is important to be able to assess the results obtained with these alternatives in order to choose the most suitable approach in each case.

Describing simple local excision with TEM for ADC-T2, Borschitz *et al*[49] reported a local recurrence rate of 35%. In our study of a series of 11 patients and a mean follow-up of 59 mo, local recurrence was recorded in 22.2%. These results suggest that local excision alone at this stage of the disease should only be used with palliative intent.

The possibilities of postoperative adjuvant therapy have received considerable attention. This is not surprising, since with adequate pathological diagnosis of the lesion (avoiding understaging or overstaging) adjuvant care can control the disease at a lower cost than radical surgery. However, the review of the literature on CT-RT after local surgery presents disappointing results, with local recurrence ranging from 0% to 45%[56]. Our experience has been unfavorable, with two out of six patients (33%) presenting local recurrence despite adequate surgical resection and with tumors defined as low risk on the strength of histological findings[15]. We agree with Baxter *et al*[56] that although postoperative adjuvant therapy appears to reduce local recurrence compared to simple local excision, the rate still remains higher than with TME.

In a review of the literature on preoperative CT-RT and local surgery in ADC-T2, Borschitz *et al*[55] observed that when complete pathological response (CPR) is achieved (that is, ypT0), local recurrence (LR) was 0% and systemic recurrence was 4%. With ypT1 tumors, LR was 2% and systemic recurrence was 7%. In ypT2, LR rose to 7% and systemic recurrence also to 7%. However, when there was no response to neoadjuvant therapy (ypT3), the LR rate was 21% and systemic recurrence

12%. Although the experience is limited, promising results have also been reported in T3, although the reports do not specify whether the lesions were superficial or deep[57,58].

The main objective of neoadjuvant treatment is to achieve CPR. The CPR rates reported for T2 and T3 range widely, between 11.7% and 73%[59,60]. The immense majority of CPRs are obtained with a long CT-RT regimen (5-fluorouracil or capecitabine, combined with radiotherapy of 50.4 Gy for five weeks). The adverse effects (AE) due to the toxicity of the CT-RT should be borne in mind. In 11 of the 40 patients (26.5%), Yu *et al*[57] reported toxicity following neoadjuvant treatment; ten of them had AE grade ≥ 2, although none abandoned treatment. In attempts to improve the CPR, adjuvant treatment regimens have been modified. Garcia-Aguilar *et al*[59] proposed the association of standard doses of capecitabine and oxaliplatin with radiotherapy, and achieved a CPR of 48%. However, 44% of patients had AE grade ≥ 3, which obliged a reduction in the capecitabine dose; with the new dose the CPR rate was 36%, and 30% presented AE grade ≥ 3.

Complete clinical response (CCR) is not always the same as CPR. Attempts have been made to determine clinical and radiological predictors of CPR, but no definitive conclusions have been reached[59,61,62]. Due to the lack of correlation between CCR and CPR, the combination of CT-RT and local surgery is not suitable for all types of rectal cancer that present CCR. For this reason, we advocate exhaustive selection of patients by a multidisciplinary team[63,64]. The most favorable results reported in the literature were in adenocarcinomas staged by EUS, MRI and abdominal CT as T2N0M0 with degrees of differentiation G1-2[50], size ≤ 4 cm, and CPR after CT-RT.

Adequate excision of the lesion is most important, avoiding fragmentation of the specimen and involvement of the surgical margins (> 1 mm)[49]. As noted above, TEM also achieves better results than conventional local excision with regard to the resection margins and the quality of the specimen[44].

The results obtained so far with TEM are promising. However, the scientific evidence is still limited, and these findings need to be confirmed in prospective randomized control trials.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (8): Gastric cancer

Racial and ethnic disparities in gastric cancer outcomes: More important than surgical technique?

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Abstract

Racial and ethnic disparities in cancer care are major public health concerns and their identification is necessary to develop interventions to eliminate these disparities. We and others have previously observed marked disparities in gastric cancer outcomes between Eastern and Western patients. These disparities have long been attributed to surgical technique and extent of lymphadenectomy. However, more recent evidence suggests that other factors such as tumor biology, environmental factors such as *Helicobacter pylori* infection and stage migration may also significantly contribute to these observed disparities. We review the literature surrounding disparities in gastric cancer and provide data pertaining to potential contributing factors.

Key words: Race; Ethnicity; Disparities; Gastric cancer; Gastric adenocarcinoma

Core tip: Our prior investigations and review of the literature suggest that racial and ethnic disparities in gastric cancer outcomes in Eastern and Western patients may not be solely attributed to surgical technique and extent of lymphadenectomy. More recent evidence from the Asian population of Los Angeles County and a broad spectrum of the United States suggests that racial disparities exist independent of the number of lymph nodes harvested. Our data suggests that gastric cancer outcomes are not comparable among different racial and ethnic groups. Therefore, a one size fits all approach to gastric cancer management appears to be inappropriate.

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INTRODUCTION

Cancer health disparities are "differences in the incidence, prevalence, mortality and burden of cancer and related adverse health conditions that exist among specific population groups"[1]. Such disparities related to race and ethnicity are well described and are major public health concerns. Indeed, cancer incidence and death rates vary considerably among select racial and ethnic groups[2,3]. For example, when considering all cancer sites combined, black men have higher incidence and death rates compared to white men; black women also have higher death rates compared to white women[2-4]. These disparities apply to much of the United States, where whites and blacks are the predominant racial groups. However, in states such as California that have a large population of immigrants, racial disparity investigations have included the Asian population.

Our research group and others have previously investigated gastrointestinal cancer outcomes in Asians

Table 1 Investigations performed by our group demonstrating racial and ethnic disparities in gastrointestinal cancer outcomes

Ref.	Histology	Cancer registry	Groups	Findings
Artinyan *et al*[5], 2010	Hepatocellular carcinoma	SEER	white, black, Hispanic, and Asian	Blacks have shortest OS Race/ethnicity independently predicts OS Asian race independently predicts improved OS
Kim *et al*[7], 2011	Rectal cancer	LAC CSP	white, black, Hispanic, and Asian	Asians have longest OS Race/ethnicity independently predicts OS
Lee *et al*[8], 2012	Rectal cancer	LAC CSP	white, black, Hispanic, and Asian	Of patients who received neoadjuvant radiation, blacks have the poorest survival Race/ethnicity independently predicts survival
Lee *et al*[9], 2012	Colon cancer	LAC CSP	white, black, Hispanic, and Asian	Asians have longest OS
Kim *et al*[16], 2010	Gastric cancer	LAC CSP	white, black, Hispanic, and Asian	Asians have longest OS Asian race independently predicts improved OS for surgical patients
Kim *et al*[59], 2009	Gastric cancer	LAC CSP	Korean, Chinese, Japanese, Filipino, and Vietnamese	Koreans have longest MS Filipinos have shortest MS Japanese and Filipino ethnicities independently predict worse OS
Nelson *et al*[18], 2013	Gastric cancer	SEER	Korean-Americans and whites	Korean-Americans have prolonged OS independent of LNs

SEER: Surveillance, Epidemiology, and End Results; LAC CSP: Los Angeles County Cancer Surveillance Program; MS: Median survival; OS: Overall survival.

revealing better survival outcomes in a variety of cancers including hepatocellular carcinoma and rectal and colon cancer[5-9] (Table 1). In addition to differences in incidence and survival, there are differences in the type of treatment received. This is more prominent in the management and subsequent outcomes of gastric cancer, one of the most common cancers in Asia[10]. Disparities in gastric cancer outcomes has been an area of active investigation, with many striving to explain the dramatic differences in survival between Eastern and Western patients. Here, we explore the literature on racial and ethnic disparities in gastric cancer and the factors that may contribute to this phenomenon.

Racial and ethnic disparities in gastric cancer

Despite a decreasing incidence in the United States, gastric cancer remains a leading cause of cancer-related death worldwide[11]. In the United States, the estimated new number of gastric cancer cases was 21600 with a corresponding estimated number of deaths of 10990[2]. Although the number of deaths from gastric cancer has been steadily declining since 1930, the disease continues to be common in Asian countries where nearly 60% of new cases occur[12]. Notwithstanding the higher prevalence of gastric cancer in Asia, significantly better outcomes have been reported in Asian compared to Western countries[13]. For example, 5-year gastric cancer survival in Japan is 60% compared to the much lower 20% in the United States and Europe[10]. However, the outcomes disparities are not limited to survival alone. In fact, important differences have also been observed in gastric cancer presentation and anatomic location and patient receipt of multi-modality therapy and surgery.

When considering disease presentation and location (proximal - cardia, fundus; distal - body, antrum, pylorus), Asian patients are more likely to be younger at initial diagnosis and to have a higher proportion of distal gastric cancers[14,15]. Our group's investigation of gastric cancer in southern California revealed that Asians, Hispanics and blacks had the lowest percentage of proximal tumors, whereas whites had the highest percentage of proximal tumors. Furthermore, Asians were more likely to have localized disease[16]. When examining receipt of therapy, our group also observed that Asians were more likely to undergo curative intent surgery[16]. Gill *et al*[15] observed that Asians also received chemotherapy more often than non-Asians. Regarding the quality of surgical resection, Al-Refaie *et al*[17] demonstrated that Asians were less likely to have inadequate lymphadenectomy compared to whites. It is no surprise then that studies have repeatedly demonstrated that Asian patients have better gastric cancer survival compared to other racial and ethnic groups[10,15,16,18]. This disparity in survival when viewed in a larger context between Eastern and Western countries has been attributed largely to surgical technique and extent of lymphadenectomy[19-21]. However, our investigations suggest that other factors such as differences in tumor biology[14,22,23] and infectious etiologies such as *Helicobacter pylori* (*H. pylori*)[24] may influence these disparities to variable extents. We discuss these factors below.

Surgical technique and extent of lymphadenectomy

One of the historical areas of controversy in the surgical management of gastric cancer is the extent of lymphadenectomy. Lymph node disease is an independent prognostic factor in gastric cancer[19,20,25,26] and prospective randomized trials have shown mixed results pertaining to the value of extended lymphadenectomy. One such study performed by the Medical Research Council in the United Kingdom[27] examined gastrectomy with D1 *vs* D2 lymph node dissection (LND) and the results showed higher morbidity and mortality in the D2 LND group.

Furthermore, 5-year overall and recurrence-free survival were not significantly different between the 2 groups[28]. The Dutch Gastric Cancer Group also conducted a major investigation, randomizing patients to undergo gastrectomy with D1 *vs* D2 LND[29]. The analysis demonstrated that D2 LND was associated with significantly greater peri-operative morbidity and mortality compared to D1 LND. Although there was no survival benefit initially observed with D2 LND[30,31], a 15-year analysis of the data showed that D2 LND was associated with lower locoregional recurrence and gastric cancer-related death rates[32]. Nevertheless, Western data is generally different from studies performed in Eastern countries. In Japan, numerous retrospective, observational, and prospective studies have shown improved survival in patients undergoing extended lymphadenectomy[19-21]. As such, D2 LND is regarded as standard of care and nearly all centers in Asia have embraced the routine performance of extended LND, whereas its performance in the United States and Western centers is likely to occur only at specialty centers.

The degree of LND is based on the Japanese staging system in which nodal stations are categorized as N1, N2, N3 or N4[19,20]. For example, D1 dissection entails removal of the N1 lymph node basin (*i.e.*, perigastric, lesser and greater curvature, suprapyloric and infrapyloric), whereas D2 dissection involves D1 dissection plus removal of nodes along the major named arteries (left gastric artery, splenic artery, common hepatic artery and celiac trunk). More extended lymph node dissections involve the removal of lymph nodes in the hepatoduodenal ligament and retropancreatic and para-aortic regions. In a randomized controlled trial comparing D1 and D3 LND, the more extensive LND was associated with higher 5-year overall and recurrence-free survival[21].

The adoption of D2 LND in Western countries has been slow and may contribute to the reported differences in survival outcomes between Eastern and Western patients undergoing surgery for gastric cancer. Interestingly, our own data suggests that the superior survival outcomes noted in Eastern populations may not be directly related to extent of lymphadenectomy. Using the National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) registry, we observed that the outcomes of Korean-American gastric cancer patients were independent of lymph node number[18]. Remarkably, despite a consistently low number of examined lymph nodes for Korean-American patients, survival rates were comparable to previously reported outcomes from East Asian centers with higher lymph node yields. These findings suggest that the higher gastric cancer survival in the East may not be attributed solely to surgical technique. However, our own group firmly adheres to the routine performance of D2 LND dissection in our patients with gastric adenocarcinoma.

Stage migration

Variability in the extent of lymphadenectomy and the number of lymph nodes examined may affect nodal staging[33-35]. Thus, the comparison of outcomes between Eastern centers with extended LND and Western centers with inadequate LND may produce disparities because of potential under-staging in Western patients. Originally described by Feinstein *et al*[36] as the Will Rogers phenomenon, there is a migration of disease into more advanced stages by the identification of lymph node disease with more extensive dissection (in Eastern patients) that would otherwise remain unidentified (in Western patients) with inadequate surgical dissection. Therefore, stage migration associated with more radical lymphadenectomy in the East may contribute to the disparate survival differences between Eastern and Western patients.

Tumor biology

The different patterns of gastric cancer in the East and West are so apparent that many have suggested inherent differences in biologic behaviour. This theory may be supported by the distinct anatomic patterns of gastric cancer location between Eastern and Western patients. Distal cancers constitute the majority of stomach cancer cases worldwide[10] whereas the incidence rates of proximal cancers have increased in Western countries[10,37,38]. While the distinct anatomic patterns and histologic subtypes of cancer may suggest differing tumor biology, studies have been unable to consistently support this notion.

From a molecular perspective, McCulloch *et al*[39,40] showed that the oncogenes *c-erbB2* and *TP53* were expressed in a similar fashion in gastric cancers from Japanese and British patients, but Theuer *et al*[23] demonstrated higher frequency of microsatellite stability in gastric cancers from Japanese compared to American patients. Theuer *et al*[22] demonstrated that normal E-cadherin expression was more common in Japanese intestinal-type gastric cancer whereas *c-erbB2* expression was higher in American gastric cancers. These findings are relevant because abnormal E-cadherin expression is associated with adverse features in gastric cancer such as loss of cell-cell adhesion (a more common feature of diffuse-type gastric cancer)[41,42] and increased *c-erbB2* expression may correlate with depth of invasion and metastasis[43]. Furthermore, tumors in British patients have a significantly greater mean cell nuclear antigen proliferation index than Japanese tumors and increased levels of an "anti-metastasis" factor have been reported in specimens from Japanese compared to British patients[44].

Different genetic backgrounds in various ethnic populations may alter susceptibility to developing gastric cancer. More recently, there has been a plethora of information pertaining to genetic polymorphisms in gastric cancer. Various genes, including but not limited to, CD14[45], glutathione S-transferase T1[46] and XRCC3[47] have been shown to alter gastric cancer susceptibility in ethnic groups, particularly Asians and Caucasians. These molecular studies suggest that differences in tumor biology among various ethnic groups exist and may contribute to racial disparities in gastric cancer outcomes.

H. pylori

Globally, *H. pylori* infection affects 50% of the worldwide population[48]. In addition to its indisputable role in chronic gastritis and peptic ulcer disease[49,50], the association between *H. pylori* and gastric cancer is also well accepted[25,50-52] and epidemiologic studies estimate that the risk of gastric cancer in *H. pylori*-infected individuals is increased by 20-fold[53]. In general, the seroprevalence rates in less developed or developing countries are higher than in developed countries[24]. Compared to the United States, Asian countries have higher seroprevalence rates and Asian immigrants have much higher rates of *H. pylori* seropositivity than whites[24,54]. Thus, the high rates of gastric cancer in Asia may occur from a complex interaction between host factors, environmental factors and *H. pylori* infection[24].

Different *H. pylori* strains occur across diverse geographic regions and differences in these strains have correlated with variations in gastric cancer epidemiology[24]. For example, cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A are major pathogenic factors that may dysregulate host intracellular signalling pathways and lower the threshold for neoplastic transformation[50]. Strains that produce CagA are more likely to cause cancer[55,56]. Finally, differences in *H. pylori* genomes have been demonstrated between East Asian and non-Asian populations[57] and may also conceivably contribute to disparities in gastric cancer outcomes.

Our research observations

Our group has taken great interest in racial and ethnic disparities in gastric cancer. Within the state of California, Los Angeles County is an ethnically diverse population in which the percentage of Asians is approximately 3-fold higher than in the general United States population and where Hispanics comprise the largest ethnic group[58]. This milieu provides a unique opportunity to study the potential association between race and ethnicity and gastric cancer outcomes. Using the Los Angeles County Cancer Surveillance Program database, we demonstrated that Asian patients with gastric cancer demonstrated longer survival than whites, Hispanics and blacks[16]. Furthermore, in patients that underwent surgical resection, superior survival was again demonstrated in Asian patients compared to whites, Hispanics and blacks[16] and remained significant even when cancer location and extent of lymphadenectomy were taken into account. Even more intriguing is the observation that there was no improvement in 5-year survival for patients with increased lymph node retrieval. Our results support the presence of persistent racial and ethnic differences despite controlling for technical factors

We subsequently compared outcomes among the different Asian ethnic groups and discovered differences in gastric cancer survival among Asian ethnicities[59]. Again using the Los Angeles County Cancer Surveillance Program database, we showed stark survival differences between Korean, Chinese, Japanese, Filipino and Vietnamese populations, with the greatest difference between Koreans and Filipinos, who had the best and worst overall survival, respectively. Korean patients were least likely to have nodal or distant disease and had a lower rate of proximal tumors; conversely, Filipino patients had amongst the highest rates of nodal and distant disease as well as proximal gastric cancers. These results suggest that there are differences in gastric cancer presentation and survival among Asian ethnicities and that combining diverse Asian ethnic populations as one single race may be grossly inappropriate.

In a more recent study utilizing the SEER registry, we compared characteristics of Korean-American patients who had high or low lymph node counts and attempted to determine whether extent of lymphadenectomy during curative-intent gastric surgery would impact survival[18]. Remarkably, overall survival was not different in Korean-American patients undergoing excision of 1-15 lymph nodes compared to 16+ lymph nodes for all stages of disease. A similar analysis was conducted for whites, which showed that overall survival diverged according to examined lymph node groups. Specifically, white patients with 16+ examined lymph nodes had significantly longer overall survival than for 1-15 examined lymph nodes for all stages of disease. These findings suggest that extent of lymphadenectomy may not contribute to survival outcomes in Eastern patients as much as previously believed and that it may be more important in Western patients.

DISCUSSION

Much literature shows that the issue of racial and ethnic disparities and cancer outcomes remains important and thus it continues to be under active investigation. These disparities are likely influenced by a number of different factors (*e.g.*, access to screening, quality of surgical care, access and response to multimodality therapy, *etc.*) and a better understanding of these disparities can lead to interventions that may help to abolish these disparities.

Stark differences in gastric cancer outcomes between Eastern and Western patients have been investigated and debated. Although many Eastern surgeons are convinced that these disparities are largely secondary to surgical technique, the importance of race and ethnicity in impacting these disparities has gained traction. As a surgical unit, we strongly advocate the routine performance of D2 lymphadenectomy for curative resection of gastric adenocarcinoma, but we also strongly suspect that factors beyond surgical control influence outcomes.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (8): Gastric cancer

Metachronous gastric cancer after successful *Helicobacter pylori* eradication

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Abstract

The high incidence of gastric cancer in Japan initially resulted in establishment of a country-wide gastric cancer screening program to detect early and treatable cancers. In 2013 countrywide *Helicobacter pylori* (*H. pylori*) eradication was approved coupled with endoscopy to assess for the presence of chronic gastritis. Current data support the notion that cure of the infection in those with non-atrophic gastritis will prevent development of gastric cancer. However, while progression to more severe damage is halted in those who have already developed, atrophic gastritis/gastric atrophy remain at risk for subsequent development of gastric cancer. That risk is directly related to the extent and severity of atrophic gastritis. Methods to stratify cancer risk include those based on endoscopic assessment of the atrophic border, histologic grading, and non-invasive methods based on serologic testing of pepsinogen levels. Continued surveillance is required because those with atrophic gastritis/gastric atrophy retain considerable gastric cancer risk even after *H. pylori* eradication. Those who have already experienced a resectable early gastric cancer are among those at highest risk as metachronous lesions are frequent even after *H. pylori* eradication. We review the role of *H. pylori* and effect of *H. pylori* eradication indicating the incidence and the predictive factors on development of metachronous cancer after endoscopic therapy of early gastric cancer. Studies to refine risk markers to stratify for risk, surveillance methods, intervals, and duration after successful *H. pylori* eradication, and whether adjuvant therapy would change risk are needed.

Key words: Atrophic gastritis; Pepsinogen; miRNA; Intestinal metaplasia; Cancer prevention

Core tip: For the patients with a history of endoscopic resection of early gastric cancer, *Helicobacter pylori* (*H. pylori*) eradication followed by continued surveillance for gastric cancer is generally required because those with severe gastric atrophy retain considerable gastric cancer risk even after *H. pylori* eradication. We review the role of *H. pylori* and effect of *H. pylori* eradication indicating the incidence and the predictive factors on development of metachronous cancer after endoscopic therapy of early gastric cancer.

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INTRODUCTION

Gastric cancer is the fourth most common cancer and second leading cause of cancer deaths worldwide with more than 700000 deaths annually[1]. There are marked geographic differences in gastric cancer incidence both within and between countries and regions with the high-

est incidence occurring in Japan, Korea, China, Eastern Europe and parts of Central and South America[2]. An attempt to reduce gastric cancer mortality led Japan in the 1950's to establish secondary cancer prevention programs to detect malignant lesions in an early and potentially treatable stage. Improved detection of early lesions using double contrast roentgenography of the stomach, gastrocamera and most recently endoscopic examination with biopsy of suspected lesions has resulted in improved survival of patients receiving operative therapy[3].

Endoscopic methods to remove premalignant as well as superficial malignant gastric lesions such as endoscopic mucosal resection and endoscopic submucosal dissection have now become the standard of care in Japan and Korea for management of early gastric cancers with no evidence of lymph node metastasis. This program has been highly successful and currently more than half of Japanese gastric cancer cases are diagnosed at an early stage[4]. Because endoscopic submucosal dissection involves removal of both the mucosa and submucosa, large lesions can be resected *en bloc* yielding an improved histopathological diagnosis compared to endoscopic mucosal resection. However, both methods result in greater post-interventional quality of life compared to surgical resection[5].

While endoscopic removal of an early gastric cancer only solves the problem of that particular lesion it does not affect the overall cancer risk. The stomach in patients with early gastric cancer typically exhibits extensive chronic atrophic changes with multiple areas with preneoplastic changes and often contains microscopic foci of intramucosal cancer[6,7]. Prior studies that examined stomachs of patients with early gastric cancer reported that detailed histologic examination will reveal foci of intramucosal cancer in up to 15%[8-10]. The presence of these lesion is likely at least partially responsible for the fact that the risk of developing a metachronous lesion following endoscopic removal of an early gastric cancer has ranged between 1% and 4% per year[8,11-13]. Because of the high risk of metachronous lesions it is recommended that these patients are enrolled in a life-long endoscopic surveillance program.

The factors that influence the rate of appearance of metachronous lesions remain unclear, however some studies have shown that *Helicobacter pylori* (*H. pylori*) eradication results in a lower risk of developing a metachronous lesion[14]. Based on prior studies and the cancer field effect, it should not be surprising that either metachronous lesion remains a risk. For example, *H. pylori* eradication in patients with *H. pylori* infection and atrophic gastritis but no evidence of cancer has been shown to reduce but not eliminate that risk suggesting a role for the organism itself, for continuing *H. pylori*-induced inflammation, or both in relation of cancer risk.

H. pylori and gastric cancer

In 1994, the International Agency for Research on Cancer (IARC) of the World Health Organization classified *H. pylori* as a definite carcinogen[15]. Two decades then passed before this knowledge was translated into the decision to approve population-wide *H. pylori* eradication for any population[7]. During this interval a number of misconceptions regarding the attributable risk of *H. pylori* infection in gastric cancer were corrected and the role of atrophic gastritis as a surrogate for cancer risk was confirmed[16]. It is now accepted that *H. pylori* infection is responsible for more than 95% of gastric cancers (*e.g.*, one study in Japan demonstrated that *H. pylori*-negative gastric cancer accounted for less than 3% among all gastric cancers)[17]. *H. pylori* infection causes progressive damage to the stomach that may eventually result in atrophic gastritis/gastric atrophy with a rapidly increasing risk of gastric cancer. It is this progressive nature of the process that makes it so dangerous and many have been lulled into compliancy when deciding what to do with a patient with mild or non-atrophic gastritis without recognizing that the current histology is actually an early stage of a progressive process and the subsequent changes are largely irreversible. However, progression can be prevented or halted by *H. pylori* eradication but the cancer risk associated with atrophic damage can at best be only partially reversed. As such, *H. pylori* infection has been likened to infestation with termites which also cause typically silent put progressive damage. As with termites, the best results are obtained when the problem is discovered before permanent and extensive damage has occurred. The failure to recognize the progressive nature of the process can result in compliancy during which an individual cancer risk progressively increases[18].

H. pylori induced gastritis is typically acquired in childhood. Initially the inflammation and damage is most severe in the non-acid secreting gastric antrum. Over time the damage progresses into the gastric corpus as an advancing atrophic border which can be recognized endoscopically, and the damage clinically progresses more rapidly along the lesser curve than the greater curve[19,20]. Chronic inflammation related with *H. pylori* affects differentiation and promotes metaplasia[21-23]. As the damage advances into the corpus along the atrophic border it leaves behind a lawn of pyloric metaplasia (also known as pseudopyloric or mucus metaplasia) now recognized to be similar or identical to spasmolytic polypeptide/trefoil factor family 2 (TFF2)-expressing metaplasia (SPEM) described in animal models of gastric cancer[24,25]. The recognition that pyloric metaplasia could be easily recognized by immunostaining as SPEM rather than the previous cumbersome process of identifying it on the basis of corpus location and pepsinogen I staining allowed many older observations to be rapidly confirmed and extended[16]. It is now believed that intestinal metaplasia arises from SPEM and SPEM may also provide the cell of origin of gastric cancer[25,26]. Intestinal metaplasia is no longer thought to be the precursor of gastric cancer but rather is an easy recognized surrogate for the presence and extent of gastric mucosal atrophy[27-29]. The concept of multifocal atrophic gastritis actually represents scat-

tered areas of intestinal metaplasia arising within a lawn of SPEM-type atrophy damage[30-32]. The ease of diagnosing pyloric metaplasia has allowed development of a new gastric cancer risk stratification system, the corpus-predominant gastritis index, to join the Operative Link for Gastritis Assessment (OLGA) and OLGA-M histology systems of stratifying gastric cancer risk[33].

SERUM MARKERS FOR GASTRIC CANCER RISK ASSESSMENT

Pepsinogen

For population wide testing it is important have efficient and cost effective practical mass screening methods that correlate with the risk of developing gastric cancer (*i.e.*, non-invasive risk stratification). Since *H. pylori* infection is the necessary but insufficient cause of gastric cancer, identification and eradication of *H. pylori* is the most important step; eradication *H. pylori* infections which will ultimately eliminate gastric cancer. There are a number of validated non-invasive methods to identify *H. pylori* infection ranging from serologic methods, through the urea breath test and stool *H. pylori* antigen testing[34]. However in populations where gastric cancer is common, *H. pylori* eradication alone is often insufficient as many individuals will have already have experienced irreversible gastric damage and thus carry an ongoing risk for development of gastric cancer despite *H. pylori* eradication. In the past when the emphasis was on identifying incidence cases of gastric cancer in high prevalence countries such as Japan, a number of approaches (secondary cancer prevention) were tested. Measurement of serum pepsinogens proved to be a useful non-invasive method of identification of patients at risk and also proved cost effective for enriching the population with gastric cancer in screening studies[35-37]. The concept is based on the fact that pepsinogen Ⅰ is produced by the chief and mucous neck cells in the fundic glands whereas and pepsinogen Ⅱ is produced throughout the stomach as well as by Brunner's glands[38,39]. Damage to the gastric corpus results in a progressive decline in both pepsinogen Ⅰ levels and the ratio of pepsinogen Ⅰ to pepsinogen Ⅱ (pepsinogen Ⅰ / Ⅱ). Pepsinogen testing thus allows a non-invasive assessment of the presence and extent of atrophic gastritis and can be used to risk identify patients endoscopic cancer screening programs or for possibly needing endoscopic surveillance after *H. pylori* eradication[6,29,35,37,40-42]. While this approach has been shown to be useful, the cumulative data have shown some limitations. Probably the most important limitation is that *H. pylori* eradication can significantly change pepsinogen levels with a decrease of pepsinogen Ⅰ and pepsinogen Ⅱ and an increase of pepsinogen Ⅰ / Ⅱ even among those at high risk for gastric cancer[43,44]. Thus, at least as currently used, serum pepsinogen testing cannot be used as a reliable marker of atrophy for patients who already have been treated by eradication therapy.

Japan has a large cadre of endoscopists experienced in detection of atrophic gastritis and early gastric cancer and the decision was made that *H. pylori* eradication therapy should be accompanied by endoscopy to examine the extent and severity of gastritis. In countries where gastric cancer risk is lower and a large number of experienced endoscopists is lacking, it would probably be more prudent and cost effective to use pre-therapy pepsinogen testing to risk stratify patients into those possibly at higher risk for subsequent gastric cancer and those with little or risk post *H. pylori* eradication. Those in the higher risk category could then undergo endoscopy using a validated risk stratification system to identify those with indications for continued surveillance.

Micro-RNA

Micro-RNAs (miRNAs) are 18-25 nucleotide noncoding RNA sequences that are transcribed but not translated into proteins. Some miRNAs have been shown to possess oncogenic or tumor suppressor activity and relate to apoptosis, proliferation, differentiation, metastasis, angiogenesis, and immune response, which are all potentially involved in cancer initiation, progression and treatment response[45,46]. MiRNAs can also be detected circulating in a cell-free form in blood, most probably in exosomes which protect them against degradation by ribonuclease, and their signatures in blood are similar in men and women, as well as individuals of different age[47,48]. Furthermore, miRNA levels are similar in plasma and serum, and freeze/thaw as well as prolonged storage at room temperature does not affect their levels[48]. Thus, serum miRNAs have the potential of a novel biomarker for many cancers. Lawrie *et al*[49] first discovered tumor-specific deregulation of circulating miRNAs and subsequently, circulating miRNAs have been suggested great potential as biomarkers for many cancers including gastric cancer[48,50,51]. Moreover, accumulating reports suggest the potential of miRNAs in the early detection of gastric cancer.

We investigated serum miRNAs as markers to individuals at high risk for gastric cancer not only before *H. pylori* eradication but also after eradication. The serum levels of miR-106b and let-7d before and after *H. pylori* eradication; miR-21 after eradication were significantly higher in the high-risk group than in controls. *H. pylori* eradication significantly changed serum pepsinogen levels even in the high-risk group, whereas eradication did not significantly alter miR-106b and let-7 levels in the high-risk group. These results suggest that serum miRNAs may be equivalent or even superior to serum pepsinogen as a biomarker to detect those at high risk for gastric cancer before and after *H. pylori* eradication[52].

EFFECT OF *H. PYLORI* ERADICATION ON CANCER INCIDENCE

In the first half of the 20th century it was recognized that gastric cancer risk was related to atrophic gastritis[7,53]. The late 20th century brought new information

and identified that gastric cancer was an inflammation-related cancer caused by chronic infection with *H. pylori*. It was initially unclear whether *H. pylori* eradication alone would suffice to eliminate or greatly reduce gastric cancer risk or whether some form of surveillance would be still required. The fact that those with atrophic gastritis whose *H. pylori* had disappeared spontaneously following destruction of the normal gastric niche for their growth still retained a high risk of gastric cancer suggested that *H. pylori* eradication alone was likely to prove insufficient[6]. Many clinical studies have subsequently examined the effect of *H. pylori* eradication on the subsequent incidence of gastric cancer. For example, Take *et al*[54] in a prospective non-randomized eradication study among more than1100 Japanese patients with peptic ulcers showed that *H. pylori* eradication reduced the risk of subsequently developing gastric cancer. A follow-up for a mean of 3.9 years of these patients found that gastric cancer developed in less frequently among those who had had successful *H. pylori* eradication compared to those with persistent infection (0.23% *vs* 0.70% at 1 year, $P = 0.04$, log-rank test)[54]. While eradication did not completely eliminate the risk, and the risk was related to the extent of atrophic gastritis at the time of eradication therapy[54]. The remained risk of developing gastric cancer was reported to be 0.30% per year[55]. The Shangdong intervention trial failed to find a difference in gastric cancer incidence after 7.3 years but did find a significant fall 14.7 years post *H. pylori* eradication therapy[7,56]. The latest meta-analysis has confirmed that successful eradication reduced the risk for gastric cancer and included 6 randomized controlled trials including four from China, one from Japan, and one from Colombia. The median follow-up period was 6 years. The pooled analysis yielded a relative risk for gastric cancer of 0.65 (95%CI: 0.43-0.89) following successful eradication therapy[57].

One effect of *H. pylori* eradication therapy is to stop the progression of damage and thus lock in or reduce the gastric cancer risk present at the time of *H. pylori* eradication[6]. Thus, those with non-atrophic gastritis would be expected to have negligible risk of subsequently developing gastric cancer whereas those with atrophic gastritis would be expected to have a risk equal to or somewhat lower than others with the same pattern of gastritis but definitely lower than an untreated cohort whose risk would increase yearly as the disease progressed[6]. The available data confirm these expectations[6,41]. However, there are few studies that have followed patients who were matched based on risk stratification. A longitudinal cohort study of 9.3 years in Japan reported significant reduction in cancer incidence after eradication in *H. pylori* positive patients with mild atrophic gastritis as evaluated by serum pepsinogen testing. The incidence per 100000 person-year in those with persistent infection was 111 compared to 69 among those the infection was eradiated. However the cancer incidence was not significantly different (237 *vs* 223) among the patients with more severe atrophy[58].

Eradication of the infection stops the inflammatory process, promotes healing of gastritis and resolution of inflammation. Nonetheless, the link between *H. pylori* and cancer runs through atrophic gastritis and intestinal metaplasia, and eradication cannot reverse the severe atrophic damage and intestinal metaplasia, especially incomplete type or SPEM that has already occurred. For examples, *H. pylori* eradication prior to development of intestinal metaplasia improves corpus gastritis enhancing sonic hedgehog (SHH) and its downstream regulators and diminishing SHH methylation and aberrant CDX2 expression, which inhibit intestinal development and differentiation and reverse gastric phenotype. However, eradication in patients with high risk such as atrophy with intestinal metaplasia, especially incomplete type or a history of endoscopic treatment for gastric cancer does not result in much if any improvement[22,59,60]. The ability to predict the point of no return for the development of the malignancy is of particular interest and whether the presence of severe atrophy, SPEM, or some intestinal metaplasia subtypes alone or together correspond to this point, still need to be investigated[61].

CUMULATIVE INCIDENCE OF METACHRONOUS CANCER AFTER ERADICATION

The group with the highest risk of gastric cancer includes those who have already had one cancer cured by upper gastrointestinal endoscopy. Several studies have reported incidence of metachronous cancer after successful *H. pylori* eradication (Table 1).

Uemura *et al*[62] were the first to show that *H. pylori* eradication could reduce the risk of development of gastric cancer in this group of patients when, in a retrospective study, 132 patients with early gastric cancer were followed after endoscopic resection; metachronous gastric cancer developed only in 6 of 67 (9%) patients without eradication over a follow up of 3 years. A later multicenter prospective randomized study in Japanese patients followed for 3 years after endoscopic removal of an early gastric cancer found metachronous gastric cancer in 9 of 272 (3.3%) patients with *H. pylori* eradication *vs* 24 of 272 (8.8%) controls[14]. The incidence of metachronous gastric cancer was reduced significantly (OR = 0.35, 95%CI: 0.16-0.78; $P = 0.009$) consistent with *H. pylori* eradication having a benefit in delaying the onset of new cancers in the same stomach.

In contrast, a Japanese retrospective study reported that *H. pylori* eradication did not reduce the incidence of metachronous gastric cancer. Baseline severe mucosal atrophy and a follow-up of more than 5 years were found to be independent risk factors for the development of metachronous gastric cancer[63]. Moreover, a recent Japanese multicenter retrospective cohort study from 12 hospitals detected metachronous multiple cancers in 65 of 1258 (5.2%) during a mean of 26.8 mo. The cumulative

Table 1 Incidence of metachronous cancer after successful *Helicobacter pylori* eradication

Ref.	Country	Subject No.	Study design	Mean follow-up periods	Incidence (%)	Eradication effect (95%CI)
Uemura *et al*[62], 1997	Japan	65/67	NR	3 yr	0 *vs* 9	effective $P = 0.011$
Fukase *et al*[14], 2008	Japan	272/272	Multicenter open-label RCT	3 yr	3.3 *vs* 8.8	effective 0.35 (0.16-0.78) $P = 0.009$
Shiotani *et al*[12], 2008	Japan	80	single arm	33 mo	11.3	
Hanaoka *et al*[66], 2010	Japan	82		55 mo	14.6	
Maehata *et al*[63], 2012	Japan	177/91	retro NR	3 yr 1.1-11.1 yr	8.5 *vs* 14.3	OR = 1.71 (0.72-4.03)
Kato *et al*[64], 2013	Japan	263/105	Multicenter retro cohort	26.8 mo 2-5 yr	3.5/Y	NS
Seo *et al*[65], 2013	South Korea	61/13	retro cohort	27.2 mo	9.8 *vs* 23.1	OR = 0.36 (0.08-1.70),
Chon *et al*[69], 2013	South Korea	129 85/44	Retro NR	26 mo 16.5-30 mo	4.7 *vs* 11.4	effective HR = 0.143 $P = 0.008$

Subject No.: Number of subjects with eradication/without eradication and with failure of eradication; RCT: Randomized controlled trial; NR: Non-randomized; Retro: retrospective; NS: Not significant.

incidence of metachronous cancers increased linearly and the mean annual incidence rate was 3.5%. The incidence rate did not differ between patients with or without *H. pylori* eradication[64]. A recent study from Korea also reported that metachronous gastric cancer showed a decrease in the eradicated group, but this did not reach statistical significance (OR = 0.36, 95%CI: 0.08-1.70, $P = 0.189$), although metachronous gastric cancer was significantly decreased in the eradicated group (OR = 0.108, 95%CI: 0.016-0.726, $P = 0.035$) among the subgroup who were followed-up for more than 18 mo[65].

These recent studies have all confirmed that the presence of one early gastric cancer identifies a group of patient at extremely high risk of metachronous cancer consistent with the histologic analysis of the remaining gastric mucosa in patients with early gastric cancer undergoing gastric resection. The risk of a metachronous cancer among those not having *H. pylori* eradication appears to be in the range of 3%-4% per year (*e.g.*, 3000 to 4000/100000 per year) (Table 1). Overall *H. pylori* eradication appears to reduce that risk but this is not seen in all studies and studies are needed to identify risk factors that correlate with subsequent risk such as pattern and extent of atrophy as well as better characterization of the mucosa in terms of inflammation, presence and extent of SPEM, different types of intestinal metaplasia, gastric microbiota, *etc.*.

PREDICTIVE FACTORS FOR METACHRONOUS GASTRIC CANCER AFTER *H. PYLORI* ERADICATION

Previous studies have looked at factors that helped predict development of gastric cancer after *H. pylori* eradication and those data might provide clues to risk stratification after endoscopic removal of an early gastric cancer and *H. pylori* eradication. *H. pylori* eradication will thus produce two populations: those with minimal to no risk and those with some residual risk for cancer. Those with residual risk can likely be assured that their risk will not increase as they age and although it will probably decrease somewhat, some risk remains[7]. Our previous study showed that atrophy in biopsy specimens from the lesser curvature of the corpus was strongly associated with gastric cancer risk[12]. The frequency of severe atrophy assessed by histology (100% *vs* 53.2%, $P = 0.03$) was higher and the serum pepsinogen I / II ratio before *H. pylori* eradication was significantly lower in the group that developed metachronous cancer compared to the group that did not. A pepsinogen I of < 25 ng/mL before eradication was significantly associated with development of a new lesion[12]. Moreover, extensive atrophic gastritis diagnosed by autofluorescence imaging (AFI), which is new endoscopic imaging technology using illumination of different wavelength light through a filter in a light source[21], was a significant predictor for metachronous cancer developed after successful eradication and could possibly be useful to identify patients undergoing endoscopic submucosal dissection who still required intensive surveillance after eradication[66].

For many individuals *H. pylori* eradication equates with cancer prevention whereas for others it only produces a reduction in risk. This difference in outcome depends on the level of risk when the eradication is performed. For those with history of endoscopic resection of one cancer, *H. pylori* eradication followed by surveillance for gastric cancer is generally indicated (*i.e.*, a combination of primary and secondary prevention), because the risk of gastric cancer remains high even after *H. pylori* eradication[6,53]. The previous study indicated that levels of hMLH1 promoter hypermethylation, which is a frequent cause of the microsatellite instability (MSI) -H phenotype, are similar in the surrounding non-cancerous tissue compared to cancer tissue[67]. In addition, another study indicated that MSI and hypermethylation of hMLH1 in cancer lesions were detected more frequently in the patients with multiple gastric cancers than those with

solitary gastric cancer[68]. These results indicate that inactivation of hMLH1 through promoter hypermethylation seems to be involved in the development of multiple gastric cancers following the MSI pathway. MSI or hypermethylation of hMLH1 as well as serum miRNA might be potential predictive markers for metachronous cancer. Studies are required to establish appropriate markers irrespective *H. pylori* eradication for gastric cancer screening.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (8): Gastric cancer

Cellular physiological approach for treatment of gastric cancer

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Abstract

Recent studies show that ion channels/transporters play important roles in fundamental cellular functions that would be involved in the cancer process. We review the evidence for their expression and functioning in human gastric cancer (GC), and evaluate the potential of cellular physiological approach in clinical management. Various types of ion channels, such as voltage-gated K^+ channels, intracellular Cl^- channels and transient receptor potential channels have been found to express in GC cells and tissues, and to control cell cycles. With regard to water channels, aquaporin 3 and 5 play an important role in the progression of GC. Regulators of intracellular pH, such as anion exchanger, sodium-hydrogen exchanger, vacuolar H^+-ATPases and carbonic anhydrases are also involved in tumorigenesis of GC. Their pharmacological manipulation and gene silencing affect cellular behaviours, suggesting their potential as therapeutic targets for GC. Our studies indicate the intracellular Cl^- concentration could act as a mediator of cellular signaling and control cell cycle progression in GC cells. Further, we demonstrate the cytocidal effects of hypotonic shock on GC cells, and indicate that the blockade of Cl^- channels/transporters enhances these effects by inhibiting regulatory volume decrease. A deeper understanding of molecular mechanisms may lead to the discovery of these cellular physiological approaches as a novel therapeutic strategy for GC.

Key words: Gastric cancer; Ion channels; Water channels; Intracellular pH; Intracellular chloride; Osmolality

Core tip: This article aims to systematically review the current knowledge on expression and functioning of ion transporters in gastric cancer (GC). Various types of ion channels, water channels and regulators of intracellular pH have been found to express in GC, and to control tumorigenesis. Our studies indicate the intracellular Cl^- concentration could control cell cycle progression in GC cells. Further, we demonstrate the cytocidal effects of hypotonic shock, and indicate that regulation of ion transport enhances these effects. A deeper understanding of molecular mechanisms may lead to the discovery of these cellular physiological approaches as a novel therapeutic strategy for GC.

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INTRODUCTION

Gastric cancer (GC) represents the second most common cause of cancer-related deaths in the world[1]. Recently,

the prognosis of GC has been improved with advances in surgical techniques, adjuvant therapy, chemoradiotherapy and molecular targeted therapy[2]. However, long-term outcomes of patients with GC remain dismal, especially for advanced disease. An improvement in the treatment of recurrent or metastatic GC depends on understanding of the molecular mechanisms regulating the tumorigenesis and the progression of the disease.

Over the past few decades, many reports have revealed that ion channels and water transporters play important roles in fundamental cellular functions. Particularly, their physiological roles in cell proliferation have been considered since cell volume changes, which require the participation of ion and water movement across the cell membrane, are indispensable in cell cycle progression. Recently, the roles of ion and water channels/transporters have been studied in cancer cells[3-7] and various types of transporters have been found in cancers of digestive organs.

This article aims to systematically review the current knowledge on expression and functioning of ion and water channels/transporters in GC cells and tissues. The ultimate objective is to evaluate the potential of cellular physiological approaches, such as regulation of ion channels, water channels, intracellular pH, intracellular ion concentration and osmolality, in clinical management of GC.

REGULATION OF ION CHANNELS

Recent studies have demonstrated that several subtypes of K^+ channels are expressed in human GC cells, and are associated with cell proliferation. Altered expression of several voltage-gated K^+ channels (Kv) has been observed in GC. Lan *et al*[8] have demonstrated that Kv1.5 protein is frequently detected in GC tissues and down-regulation of the expression of Kv1.5 in SGC7901 cells inhibits their proliferation and tumorigenicity. Further, Han *et al*[9] have shown that up-regulation of Kv1.5 increases the K^+ current density and sensitivity of SGC7901 cells to multiple chemotherapeutic drugs, such as adriamycin or 5-fluorouracil. Expression of Kv4.1 has been found in human GC cell lines, and its down-regulation inhibited cell proliferation via the blockage of G_1-S transition[10]. Eag1 (Kv10.1) was aberrantly expressed in GC tissues and associated with cancer lymph node metastasis and stage[11]. Human ether-a-go-go-related gene (HERG) encodes one of the components of delayed rectifier K^+ currents. In GC, HERG channel has cancer-limited expression and its blocker diminishes the G_1-S transition[12,13]. Ding *et al*[14] have shown that the survival rates for the hERG1-positive expression group are significantly lower than the negative group, and hERG1 expression is found to be an independent prognostic factor. Further, HERG expression has been reported to be essential for cisplatin to induce apoptosis in human GC[15]. Disruption of K^+ channel protein, voltage-gated K^+ channel subfamily E member 2 (KCNE2), has been shown as a possible risk factor for gastric neoplasia[16]. Kuwahara *et al*[17] have analyzed the expression of KCNE2 in surgically excised tissue from human GC associated with gastritis cystica profunda and confirmed that reduced KCNE2 expression correlates with disease formation. It has been proposed that atrial natriuretic peptide modulates the proliferation of human GC cells *via* voltage-gated K^+ channels, KQT-like subfamily member 1 (KCNQ1)[18]. Inwardly rectifying K^+ channels (K_{ir}) have been also implicated in GC. Lee *et al*[19] have demonstrated that knockdown of Kir2.2 suppresses tumorigenesis by inducing reactive oxygen species-mediated cellular senescence.

There is evidence also for Cl^- channels involvement in GC. Overexpression of chloride intracellular channel 1 (CLIC1) is shown to be a potential prognostic marker for GC[20]. Elevated CLIC1 expression is strongly correlated with lymph node metastasis, lymphatic invasion, perineural invasion and pathological staging, suggesting that it is a potential prognostic marker[20]. Zheng *et al*[21] have shown that PA28β regulates cell invasion of GC by modulating the expression of CLIC1. On the other hand, Ma *et al*[22] have shown that high CLIC1 expression inhibits proliferation and enhances apoptosis, migration and invasion of GC cells.

The transient receptor potential (TRP) superfamily consists of a highly diverse group of ion channels that are mostly permeable to monovalent and divalent cations. TRP channels may be divided into seven subfamilies, including the classical (TRPC), the vanilloid receptor related (TRPV) and the melastatin related (TRPM) channels. In GC, Cai *et al*[23] have shown that Ca^{2+} elevation regulated by TRPC6 channels is essential for G_2/M phase transition and suppresses growth in human GC cells. TRPV6 has been implicated in capsaicin-induced apoptosis in GC cells[24]. Further, several reports have shown important roles of TRPM7 in apoptosis and cell viability of GC cells[25-28].

There is significant evidence for involvement of these ion channels in GC cell proliferation and disease progression. Hence, their clinical potential would be worth investigating further.

REGULATION OF WATER CHANNELS

Aquaporins (AQPs) are transmembrane proteins that facilitate transport of water and, in some cases, small solutes across membranes; charged species are not permeated. Thus, AQPs are important for cell volume regulation and electrolyte balance under both physiological and pathophysiological conditions. To date, 13 AQP subtypes and their pathophysiologic roles have been characterized in humans. In GC, several reports indicated the role of AQP3 in signal pathway. Huang *et al*[29] have shown that AQP3 plays a critical role in human epidermal growth factor (hEGF) -induced cancer cell migration and proliferation and that hEGF induced AQP3 expression via ERK signal transduction pathways. Wang *et al*[30] have demonstrated that c-Met regulates the expression of

AQP3 via the ERK signalling pathway in GC. Xu *et al*[31] have shown that AQP3 positively regulates matrix metalloproteinases (MMPs) proteins expression through the PI3K/AKT signal pathway in human GC cells. Recently, the microRNA-mediated gene repression mechanism involved in AQP3's role has been investigated in GC[32]. AQP5 also plays an important role in the tumorigenesis, progression and differentiation of human GC cells[33,34]. Shen *et al*[35] have reported expression profiles of multiple AQPs in human GC and their clinical significance. AQP3 and AQP5 are detected remarkably more strongly in carcinoma tissues than in normal mucosa by immunofluorescence. They have shown that both AQP3 and AQP5 expression are associated with lymph node metastasis and lymphovascular invasion in patients.

These results indicate that AQPs play important roles in the tumorigenesis and progression of human GC and suggest that, especially, AQP3 and 5 can become potential therapeutic targets against GC.

REGULATION OF INTRACELLULAR PH

Anion exchanger (AE) proteins facilitate the electroneutral exchange of Cl^- for HCO_3^- across the plasma membrane of mammalian cells and thus contribute to regulation of intracellular pH. The AE family is now comprised of three members, AE1, AE2 and AE3. AE1 is frequently expressed in GC, where it fails to traffic to the plasma membrane, but interacts with the tumor suppressor p16 in the cytoplasm. Down-regulation of AE1 in gastric cancer SGC7901 cells is shown to inhibit cell growth and clinical analyses have indicated that AE1 expression is associated with a low survival rate of GC patients. Suppression of AE1 induces cell death in human GC cells[36-39]. Expression of AE2 in human GC has been also investigated, and AE2 is associated with gastric carcinogenesis and achlorhydria[40]. Wang *et al*[41] have shown that early growth response protein 1 is critical for gastrin-dependent up-regulation of AE2 in GC cells.

The sodium-hydrogen exchanger (NHE) mediates a coupled counter-transport of one H^+ ion in exchange for one Na^+ ion. The basic role is to maintain intracellular pH, but NHE proteins are also important for regulation of cell volume and growth. Liu *et al*[42] have shown that the NHE1 antisense gene significantly suppresses cell growth and induced cell apoptosis in SGC7901 cells. Nagata *et al*[43] have shown that rapid and extensive decrease of intracellular pH caused by NHE1 inhibitors leads MKN45 and MKN74 cells to apoptotic and cytotoxic events.

Vacuolar H^+-ATPases (V-H^+-ATPases), as the specific proton pump of the cell, play an important role in maintaining intracellular pH. Proton pump inhibitors (PPI), mainly treating acid-related diseases, inhibit the expression of V-H^+-ATPases. Chen *et al*[44] have shown that PPIs decreased the intracellular pH of SGC7901 cells, by inhibiting V-H^+-ATPases, and enhanced the cytotoxic effects of antitumor drugs.

The carbonic anhydrases (CAs) are a family of zinc metalloenzymes that have an important role in cellular pH regulation through reversible hydration of carbon dioxide to carbonic acid. To date, 16 isozymes have been identified, which differ in tissue distribution, subcellular localization, and catalytic activity. Expression of CA IX has been found at the invasion front of gastric cancers[45]. Kato *et al*[46] have shown that the CA IX expression level is significantly high in cases of type 4 GC and diffuse type GC, and significantly correlates with the invasion depth in lymph node metastasis. The prognosis for CA IX-positive patients is significantly poorer than that of CA IX-negative patients.

These results suggest that pH regulators, such as AEs, NHEs, V-H^+-ATPases and CAs are potentially key therapeutic targets and the silencing of their expression could provide a new therapeutic approach for treating GC.

REGULATION OF INTRACELLULAR ION CONCENTRATION

Several reports indicating the important roles of Cl^- channels/ transporters on cell proliferation suggest that the intracellular chloride concentration ($[Cl^-]_i$) regulated by them would be one of the critical messengers. We have investigated roles of the $[Cl^-]_i$ in cell cycle progression of human GC cells[47]. We have found that furosemide, a blocker of $Na^+/K^+/2Cl^-$ cotransporter (NKCC), diminished cell growth by delaying the G_1-S phase progression in GC cells with high expression and activity of NKCC[48]. NKCC is one of the important transporters controlling the $[Cl^-]_i$ *via* uptake of Cl^- into the intracellular space and, therefore, furosemide decreases the $[Cl^-]_i$[49]. Cl^- channels also contribute to the regulation of $[Cl^-]_i$ which is related to cell volume. When cell shrinkage occurs isosmotically, $[Cl^-]_i$ decreases because the major membrane-permeable anion is Cl^-[50]. Furthermore, we have found that the decrease of the $[Cl^-]_i$ inhibits cell growth of GC cells and that this inhibition of cell growth is due to cell cycle arrest at the G_0/G_1 phase caused by diminution of CDK2 and phosphorylated Rb[51]. The decrease of the $[Cl^-]_i$ significantly increased expressions of p21 mRNA and protein[51]. In addition, we revealed that the $[Cl^-]_i$ affects cell proliferation *via* activation of MAPKs through up-regulation of p21 in GC cells[52]. Similar phenomena are also observed in GC cells with low $[Cl^-]_i$ caused by inhibition of NHE[53]. These findings suggest that the $[Cl^-]_i$ regulates important cellular functions in GC cells, leading to the development of novel therapeutic strategies.

REGULATION OF OSMOLALITY

Several previous studies have indicated the cytocidal effects of hypotonic stress on cancer cells. Lin *et al*[54] have reported that peritoneal lavage with distilled water improves the survival rate in patients with spontaneously ruptured hepatocellular carcinoma. Huguet *et al*[55] have discussed the optimal method for peritoneal lavage with

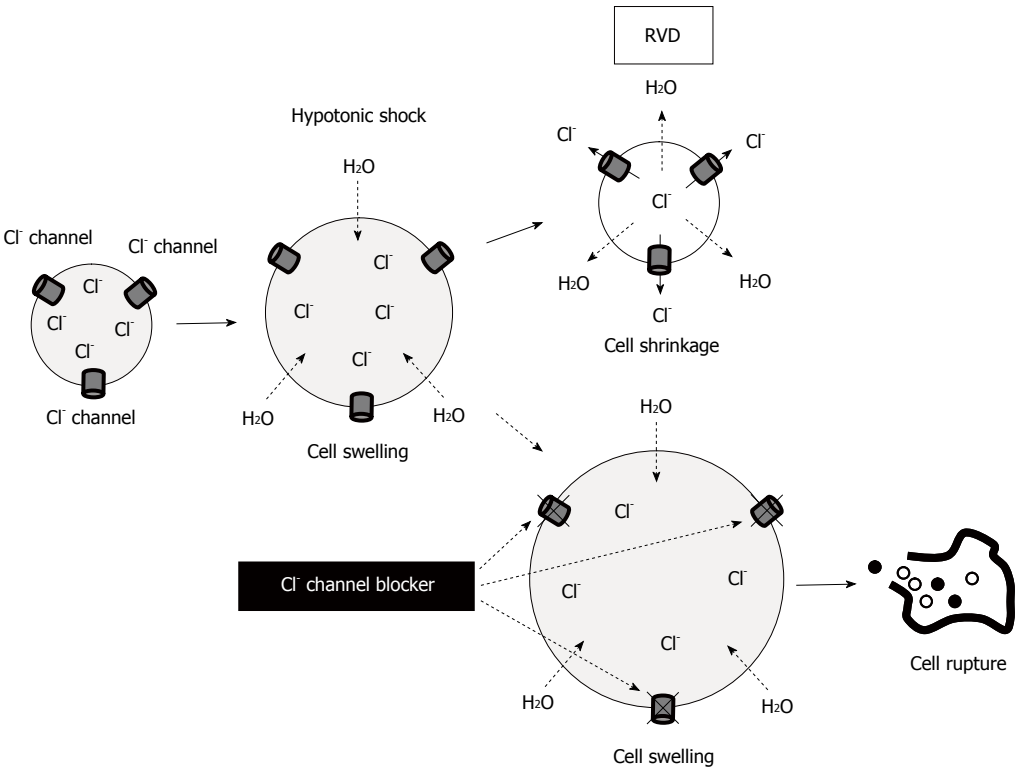

Figure 1 Blockade of Cl^- movement (channel) enhances the cytocidal effect of hypotonic solution *via* the inhibition of regulatory volume decrease in cancer cells. RVD: Regulatory volume decrease.

distilled water during colorectal cancer surgery. In GC, Mercill *et al*[56] have reported that exposure to distilled water reduces the number of surviving gastric cells. Tsujitani *et al*[57] have shown that hypotonic intraperitoneal cisplatin treatment with distilled water at the time of a gastric resection is well tolerated for patents with GC. Recently, we have analyzed the changes in the cellular morphology and volume of GC cells subjected to hypotonic stress using several unique methods and apparatus, such as a differential interference contrast microscope connected to a highspeed digital video camera and a high-resolution flow cytometer[58]. Video recordings by high-speed digital camera have demonstrated that hypotonic shock with distilled water induces cell swelling followed by cell rupture. Measurements of cell volume changes using a high-resolution flow cytometer indicate that severe hypotonicity with distilled water increases broken fragments of GC cells within 5 min. In addition, we treated the GC cells with 5-nitro-2-3-phenylpropylamino)-benzoic acid (NPPB), a Cl^- channel blocker, to enhance the cytocidal effects of the lavage by increasing their cell volume during hypotonic stress via the inhibition of regulatory volume decrease (RVD)[59,60]. RVD occurs after hypotonicity-caused cell swelling. RVD is caused by activation of ion channels and transporters, which cause effluxes of K^+, Cl^-, and H_2O, leading to cell shrinkage. NPPB is the broad spectrum Cl^- channel blocker which is fat-soluble and inhibits both Cl^- channels in cell membrane and CLIC. In MKN45 and Kato-Ⅲ cells, treatment with NPPB increases cell volume by inhibiting RVD and enhances the cytocidal effects of the hypotonic solution (Figure 1). We have found similar phenomena in esophageal[61] and pancreatic cancer cells[62]. AQPs also contribute to RVD[63]. On the other hand, NKCC plays some roles in regulatory volume increase (RVI)[64].

These findings demonstrate the cytocidal effects of hypotonic shock on GC cells, and suggest that the regulation of ion transport enhances these effects. A deeper understanding of ion transport mechanisms in gastric cancer cells during hypotonic shock could lead us to the development of novel therapeutic strategies.

CONCLUSION

This review shows a variety of ion channels, AQPs and pH regulators are expressed in human GC cells and tissues. Their expression relates to the pathological character of the GC tissues. Pharmacological manipulation and gene silencing affect their activities and fundamental cellular functions that would be involved in the GC process. Overall, we can suggest that ion, water channels and pH regulators are functional biomarkers and therapeutic targets in GC. A deeper understanding of molecular mechanisms may lead us to the discovery of these cellular physiological approaches, such as regulation of ion channels, water channels, intracellular pH, intracellular ion concentration and osmolality, as a novel therapeutic strategy for GC.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (8): Gastric cancer

What make differences in the outcome of adjuvant treatments for resected gastric cancer?

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Abstract

After a long history of Dark Age of adjuvant chemotherapy for gastric cancer, definite evidences of survival benefit from adjuvant treatment have been reported since 2000s. These survival benefits are likely attributed to something new approach different from pervious studies. In 2001, South West Oncology Group INT0116 trial yielded survival benefit in curatively resected gastric cancer patients with postoperative chemoradiotherapy [5-fluorouracil (5-FU) + Leucovorin + radiotherapy], followed by positive result by MAGIC Trial, employing perioperative(pre- and postoperative chemotherapy with Epirubicin, cisplatin (CDDP), 5-fluorouracil (ECF) regimen in patients with curative resection. A novel drug [S1: ACTS-GC (Adjuvant chemotherapy trial of TS-1 for gastric cancer) in 2007], or new drug combination chemotherapys [CDDP + 5-FU: FNCLCC/FFCD (Federation Nationale des Centres de Lutte contre le cancer/Federation Francophone de Cancerologie Digestive) in 2011, Capecitabine + Oxaliplatin: CLASSIC in 2012] also produced positive results in terms of improved prognosis. Neoadjuvant or perioperative chemotherapy, novel anti-cancer drugs, and chemoradiotherapy might be the key words to develop further improvement in the adjuvant treatment of resectable gastric cancer. Moreover, it is not new but still true to stress the importance of D2 surgery as the baseline treatment in order to minimize the amount of residual tumor after surgery.

Key words: Resected gastric cancer; Phase Ⅲ clinical trial; Adjuvant and neo-adjuvant therapy; Chemoradiotherapy; Review

Core tip: Recent positive results of adjuvant clinical trials for gastric cancer are attributed to new approaches different from previous negative trials. Inclusion of novel effective drug (S-1: ACTS-GC) and new combination of drugs (capecitabine and oxaliplatin: CLASSIC/Cisplatin and 5-fluorouracil: FNCLCC/FFCD), combination of chemotherapy and radiotherapy (SWOG INT0116), and combination of different timing (pre- and postoperative: MAGICC), might have contributed to yield positive results after curative D2 surgery. D2 surgery is going to be adopted as recommended treatment in Eastern and Western countries, and should be the baseline treatment to minimize the amount of residual tumor in future trials of adjuvant treatment.

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INTRODUCTION

Previous review articles on the adjuvant treatment for gastric cancer have commonly stated that the prognosis of gastric cancer patients still remains poor even after

Table 1 Recent epoch making adjuvant trials showing survival benefit

Reporter/group	Regimen	Patients	3 ysr/ treated	3 ysr/ control
Macdonald/2001 (SWOG INT0116)	FL + (Rad. + FL)	Curat. D2	50	41
Cunningham/2006 (MAGIC)	EPF (peri-op.)	Curat.	36[1]	23[1]
Sakuramoto/2007 (ACTS-GC)	S-1	Ⅱ-Ⅲ	80.1	70.1
Ychou/2011 (FNCLCC/FFCD)	CDDP + 5-FU (peri-op.)	Curat.res.	38[1]	24[1]
Bang/2012 (CLASSIC)	Cape. + Oxal.	Ⅱ-ⅢB	74	51

[1]Five year survival rate. 3 ysr: 3 year survival rate (%); FL: 5fluorouracil + leucovorin, Rad.: Radiation; Curat.: Curative; EPF: Epirubicin + CDDP + 5-FU; Cape.: Capecitabine; Oxal.: Oxaliplatin; peri-op: Peri-operative.

curative resection was performed, and that adjuvant treatment almost failed in improving the prognosis of gastric cancer patients[1-3]. However, in resent years, a few clinical trials reported a certain survival benefit from adjuvant treatments[4-7]. What make differences in the outcome of adjuvant treatment for resected gastric cancer? Possible reasons may be attributed to the emergence of new effective drugs, new combination of chemotherapy, radiation or hyperthermia, different delivery timing (pre-, post- or perioperative) or route of drugs (intravenous, intra-arterial, or intra-peritoneal) which might increase the local drug concentration at tumor sites, less amount of residual tumor due to improved surgical technique, modified surgical indication for advanced diseases, or elaborate trial design, or increased compliance of drug administration.

Recent improvement in adjuvant treatment has been evaluated by the literature review in relation to the factors mentioned above with an aim of getting promising suggestions for future trials.

LITERATURE RESEARCH

Evaluation of treatment effect needs proper selection of endpoints. Survival benefit is principally evaluated by the phase Ⅲ trials with overall survival (OS), relapse free survival (RFS), or median survival time (MST) as primary endpoints. On reviewing the previous literatures through electronic database (PubMed), or referring to previous systemic reviews, inclusion criteria are as follows: (1) prospective randomized phase Ⅱ or Ⅲ trials with sufficient number of patients based on the statistics, elaborated analysis based on well-managed data, and clear conclusions as to the survival benefit in relation to the endpoints stated above; and (2) literatures published later than 2000 with a few exception for important reports published before 2000.

RESULTS AND DISCUSSION

Recent state of the arts in adjuvant treatments of resected gastric cancer

Adjuvant chemotherapy had failed in improving the prognosis of gastric cancer patients until the report of SWOG INT-0116 trial[4] with adjuvant chemoradiotherapy in 2001 (Table 1). This is the first report that the large-scale phase Ⅲ clinical trial in patients with curatively resected gastric cancer yielded the significant survival benefit from postoperative adjuvant chemoradiotherapy. Combination of chemotherapy (5-fluorouracil and leucovorin: FL) and radiotherapy + FL, a new approach to the adjuvant treatment, might decreased the amount of residual tumor after surgery. Although there are several criticisms that the results of this study could not be extrapolated to Asian countries where surgery alone produced better survival in curatively resected patients, the chemoradiotherapy has become the standard treatment of locally advanced gastric cancer in the United State. Clinical significance of postoperative chemoradiotherapy is to compensate underpowered surgery (D0 surgey in 54%)[3,8]. This success was followed by a perioperative adjuvant chemotherapy with ECF (epirubicin + cisplatin + 5-fluorouracil) in patients with curatively resected gastric cancer (MAGIC Trial)[9] in 2006. This treatment is new in terms of the combination of pre- and postoperative adjuvant chemotherapy. Comparing with ordinary postoperative chemotherapy, neoadjuvant chemotherapy has some theoretical advantages[10,11]: relatively high dose intensity available, down-staging of the tumor, eradication of micro-metastasis, reduction of residual tumor burden after curative surgery. Postoperative chemotherapy might also diminish minimal residual tumor after surgery. Low dose-compliance of this postoperative chemotherapy was the target of criticism, but it has become an European standard for locally advanced resectable gastric cancer. In 2007, a large-scale clinical trial in Japan compared S-1 (combined drug of tegafur, gimeracil, oteracil potassium) plus D2-surgery and D2-surgery alone in patients with Stage Ⅱ or Ⅲ gastric cancer, and reported significant survival benefit at the median follow-up time of 3 years (ACTS-GC trial)[5]. S-1 is a new oral drug: tegafur (5-fluorocuracil derivative), gimeracil (an inhibitor of dihydropyrimidine dehydrogenase) and oterasil (inhibitor of orotate phosphoribosyltansferase), and it is reported to be highly active with mild toxicities in advanced gastric cancer[12,13]. In 2011, another perioperative adjuvant chemotherapy with Cisplatin and 5-fuorouracil also reported a significant survival benefit in patients with curatively resected lower esophageal, esophago-gastric junction, and gastric cancer. The two drugs themselves were not new, but they were used before and after surgery (FNCLCC/FFCD)[6]. A new combination of adjuvant capecitabine and oxaliplatin (Adjuvant capecitabine and oxaliplatin for gastric cancer after D2 gastrectomy: CLASSIC Trial)[7] also yielded a significant survival benefit at 3 years after surgery in patients with stage Ⅱ-ⅢB gastric cancer after D2 surgery in Korea in 2012. Survival benefit in both ACTS-GC and CLASSIC trials continued later than 5 years after surgery[14]. Thus, these successful trials seems to commonly share something new in terms of a new drug or new combination regimen, or pre- and postoperative adjuvant chemotherapy, or a new combined

Table 2 Postoperative adjuvant chemotherapy

Reporter/year	Regimens *vs* Surgery alone	Patients	OS 5ysr (%)	
			Treated	Control
Nakajima/1984	MFC + F/MF′ C + F′	Curat. Res.	68.4/62.5	51.4
Coombes/1990	FAM	Curat. Res.	45.7	35.4
Krook/1991	FA	Curat. Res.	32	33
Macdonald/1995	FAM	Ⅰ-Ⅲ	37	32
Lise/1995	FAM2	Ⅱ, Ⅲ	42 mo[1]	36 mo[1]
Grau/1998	MMC + FT	Curat. Res.	67[2]	45
Nakajima/1999 (JACOG8801)	MF + UFT	S(+)	85.8	82.9
Neri/2001	ELF	N(+)	30.2[2]	12.6
Bajetta/2002	ELF	N(+)	52	48
Nashimoto/2003 (JCOG9206-1)	MFC + F	Curat. Res. S(-)	91.2	86.1
Chipponi/2004	FLP	S(+) or N(+)	39	39
Bouche/2005 (FFCD)	FP	Ⅱ-Ⅳ	46.6	41.9
De Vita/2007 (GOIM 9602)	ELFE	ⅠB-ⅢB	48	43.5
Nakajima/2007 (NSAS-GC)	UFT	T2/N1−2	86[2]	73
Di Costanza/ 2008 (GOIRC)	PELF	ⅠB-Ⅳ	47.6	48.7
Sasako/2011 (ACTS-GC)	S-1	Ⅱ-Ⅲ	71.7[2]	61.1
Miyashiro/2011	CDDPip + CFiv	S(+), curative	62	60.9
Noh/2013 (CLASSIC)	Cape. + Oxal.	Ⅱ-ⅢB	78[2]	69
Kang/2013 (AMC0201)	MMC + 5′ FUDR (MF′) *vs* MF′ + CDDP	Ⅱ-Ⅳ	61.1	57.9
Lee/2012 (ARTIST)	Cape. + CDDP(XP) *vs* XP + Rad. + XP	Curative, D2	78.2[3]	74.2

[1]Median survival time (month); [2]Statistically significant; [3]Three year Disease free survival. OS 5ysr: Overall 5 year survival rate; MFC/F: Mitomycin C (MMC) + 5-FU + Cytosine arabinoside(CA) iv, and oral 5-FU; MF′C/F′: MMC + Ftoraful (FT) + CA iv and oral ftoraful; FAM: 5-FU + Adriamycin + MMC; FA: 5-FU + Adriamycin; FAM2: Modified 5-FU + Doxorubicin + MMC; MF+UFT: MMC + 5-FU iv and oral UFT; ELF: Etoposide + Leucovorin + 5-FU; FLP: 5-FU + Leucovorin + CDDP; FP: 5-FU + CDDP; ELFE: Epirubicin + Leucovorin + 5-FY + Etoposide; PELF: 5-FU + Leucovorin + CDDP + Epidoxorubicin; CDDPip + CFiv: CDDP ip + CDDP + 5-FU iv; S(+): Serosa involved; Curat. Res.: Curative resection.

modality of chemotherapy and radiotherapy after high compliance D2 surgery.

What have made differences in the outcome of adjuvant treatments?

As stated above, reviewing previous papers is important to elucidate the reasons of obtaining survival benefit.

Type of surgery: Incomplete surgery does not produce permanent cure, and the treatment results seems inversely correlated with the amount of residual tumor behind surgery. There is no definite evidence for D2 superiority to D1[15], but D2 surgery has been accepted in recent adjuvant trials as base-line treatment. This is the reason why curative surgery (D2) combined with appropriate adjuvant treatment is the minimum requirement for better treatment results[16]. Since 1960s in Japan, D2 surgery has been adopted as the standard treatment choice in primarily resectable cancer[17]. D2 surgery has been accepted not only in Eastern but also in Western countries (NCCN and EORTC guidelines: http://www.nccn.org/professionals/physician_gls/f_guidelines.asp; http://www.medscape.com/viewarticle/751024).

Table 3 Phase Ⅲ Neoadjuvant chemotherapy trials

Reporter/year	Regimen	Patients	Survival benefit	
			Trial	Control
Songun/1999	FAMTX	Curat. Res.	15/27[1]	18/29
Stahl/2009	PLF + rad. *vs* PLF	Curat. Res. (E-G J)	47.4[2]	27.7
Schumacher/2010	PL	Curat. Res.	72.7[3]	69.9

[1]R0 surgery rate; [2]Three year survival rate, P = NS; [3]Two year survival rate. FAMTX: 5-FU + Adriamycin + Methotrexate; PLF + rad.: CDDP + Leucovorin + 5-FU and radiation; E-G J: Esophago-gastric junction; Curat. Res.: Curative resection.

Postoperative chemotherapy with new drugs: Curative resection and postoperative adjuvant chemotherapy has been the ordinary treatment modality for a long time. Table 2 shows the summary of recent phase Ⅲ trials of postoperative chemotherapy[14,18-34]. Most of adjuvant trials reported around 2000 were using mitomycin C-, 5-fluorouracil- or Adriamycin-based regimens, most of which failed to produce survival benefit. Statistically significant survival benefit was reported in a trial done before 2000[35], but the sample was too small to confirm the survival benefit. However, a new drug such as S-1, or a new combination such as Capecitabine and Oxaliplatin yielded positive results in terms of survival benefit in the ordinary settings of postoperative adjuvant chemotherapy. It suggests that previous treatment failure is not always attributed to the trial setting itself, but to ineffective drugs. Clinicians' pessimism in 1990s led statisticians to make meta-analysis of phase Ⅲ trials of adjuvant chemotherapy[36-41], which somehow encouraged clinicians by showing a marginal survival benefit, and at the same time caused a paradigm shift from postoperative to neoadjuvant chemotherapy in 1990s[3,42,43].

Neoadjuvant chemotherapy: According to the delivery routes of drugs, neoadjuvant chemotherapy employs systemic[44-49], intra-arterial (ia), or intra-peritoneal (ip) delivery, or combination of different deliveries. Though numerous small size phase Ⅱ trials have been reported in 1990s, but large scale phase Ⅲ trials are not so much done as shown in Table 3[48,50]. It is because that neoadjuvant chemotherapy has generally been indicated to patients with high risk of relapse. Ia or ip chemotherapy has advantage to increase in the local drug concentration at the tumor site[51]. Ia chemotherapy through hepatic artery has been used to control hepatic metastasis, and sometimes induce drastic tumor shrinkage. Ip chemo-

therapy is sometimes done at the time of surgery, or *via* a catheter placed into the peritoneal cavity connected with a delivery device implanted in the subcutaneous tissue[52]. These local delivery methods rarely be done in single use, but they were combined with systemic administration (iv or oral), and ip administration was sometimes combined with hyperthermia[53].

Combination of different delivery timings, or delivery routes: Past postoperative adjuvant chemotherapy failed to improve the prognosis, but as stated before, MAGIC trial used a combined regimen of pre-and postoperative (peri-operative) adjuvant chemotherapy regimen and succeeded to yield survival benefit. Referring to the result of INT 0116 study and Magic trial, a phase Ⅱ trial of combined preoperative chemotherapy and radiotherapy was done for locally advanced gastric cancer with favorable results[54], and a phase Ⅲ trial by EORTC (CRITICS) is now conducted to determine which chemotherapy or chemoradiotherapy should be chosen in postoperative treatment[55]. Combination of systemic and local delivery (ia or ip chemotherapy) is sometimes employed in adjuvant or neoadjuvant settings[32,56-63]. Some of them produced favorable results in terms of local effect and survival benefit. Ia chemotherapy was seldom used as neoadjuvant setting for resectable gastric cancer, but was sometimes applied to patients with unresectable diseases such as large T4, liver metastasis, or peritoneal dissemination[64-66]. The author tried a combined systemic and ia chemotherapy[67] (FLEP regimen: 5-FU + leucovorin, systemic, and etoposide + CDDP, ia) for preliminarily unresectable gastric cancer associated with hepatic metastasis, paraaortic lymph node metastasis, or peritoneal dissemination. Response rate was 50%, and some of responders were subjected to radical surgery, and survived more than 5 years. Phase Ⅱ trials generally reported good local response which sometimes resulted in prolonged survivals, but survival benefit of neoadjuvant chemotherapy had not been confirmed by phase Ⅲ setting until Magic trial.

Clinical response to chemotherapy is reportedly correlate with postoperative survival[43,68], but histologic response to chemotherapy was not always the surrogate endpoint of better survival[46,69,70].

Improvement in the methodology of clinical trial: Clinical trial design has been elaborated during the long history of adjuvant treatments. Proper selection of subjects, appropriate sample size, adequate endpoints, adherence to protocol, elaborate follow-up schedule and excellent data management, proper interim and final analyses are important elements to carry out high quality clinical trials.

Previous trials often failed to show positive results in terms of survival benefit due to underpowered sample size. Recent collaboration between physicians and statisticians has opened the right way to statistical basis of clinical trials.

Five-year survival rate has been employed as the true endpoint of survival benefit in the past trials. In recent trials such as ACTS-GC or CLASSIC trials, three-year survival rate was employed as the early predictor of survival benefit based on the result of interim analysis. Later follow-up study revealed that the survival benefit has been kept later than 5 years.

In AVAGAST trial[71], significant survival benefit was observed by RFS (relapse free survival), but not by overall survival (OS). OS is influenced by the effect of subsequent treatment, but RFS is not. It is a difficult issue whether to choose RFS or OS as the primary endpoint, but recent trials tend to choose RFS.

Future perspective

Recent positive results in adjuvant treatment of gastric cancer after resection have encouraged physicians to develop further effective treatments. It may be rational and efficient way to employ in adjuvant setting a drug or combination of drugs which yielded good response in advanced gastric cancer. Combination of chemotherapeutic drugs and molecular targeting drugs, such as trastuzumab[28], bevacizusmab[71], or apatinib[72] may be promising in adjuvant trials.

Determination of proper duration of adjuvant chemotherapy[49] is another important issue in a positive regimen. It is important from the point of patient' quality of life, and medical economics, if shorter or less aggressive regimens are equivalent or superior to standard regimen.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (8): Gastric cancer

Perspectives on new biomarkers in gastric cancer: Diagnostic and prognostic applications

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Abstract

Gastric cancer is considered one of the most deadly tumors worldwide. Even with the decline in its incidence, the mortality rate of this disease has remained high, mainly due to its late diagnosis and to the lack of precise prognostic markers. The main purpose of this review is to present genetic, epigenetic and proteomic molecular markers that may be used in a diagnostic and prognostic manner and to discuss the pros and cons of each type of marker for improving clinical practice. In this sense, we observed that the use of genetic markers, especially mutations and polymorphisms, should be carefully considered, as they are strongly affected by ethnicity. Proteomic-based markers show promise, but the higher costs of the associated techniques continue to make this approach expensive for routine use. Alternatively, epigenetic markers appear to be very promising, as they can be detected in bodily fluids as well as tissues. However, such markers must be used carefully because epigenetic changes may occur due to environmental factors and aging. Despite the advances in technology and its access, to date, there are few defined biomarkers of prognostic and diagnostic use for gastric tumors. Therefore, the use of a panel of several approaches (genetic, epigenetic and proteomic) should be considered the best alternative for clinical practice.

Key words: Molecular markers; Epigenetic; Genetic; Proteomic; Diagnosis; Prognosis; Gastric tumors

Core tip: Despite the advances in technology and its access, to date, there are few defined biomarkers of prognostic and diagnostic use for gastric tumors. Therefore, a combination of several approaches (genetic, epigenetic and proteomic) should be considered the best alternative for clinical practice. Considering this point of view, this review aims to discuss the most studied biomarkers, discussing the pros and cons of each type of marker and their use in the clinical practice.

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INTRODUCTION

Despite the decline in its incidence since World War Ⅱ,

gastric cancer remains the fourth most common type of cancer worldwide and is tied with lung cancer as the second leading cause of death from cancer[1,2]. The global incidence of gastric cancer for 2008 was estimated to be 989000 new cases and 738000 related deaths[3].

It is known that gastric cancer is a multifactorial disease involving environmental factors, such as *Helicobacter pylori* infection, and genetic susceptibility[4,5]. Histologically, gastric cancer is classified according to Làuren[6] into two types, diffuse (or undifferentiated) and intestinal (or differentiated), with the majority of cases being of the intestinal type[7].

Despite improvements in medical technology, such as the development of new diagnostic imaging methods, gastric cancer remains a silent disease that is frequently diagnosed in advanced stages, which is responsible for its elevated mortality[8]. Additionally, the presence of metastasis in the lymph nodes is a frequent event in this type of neoplasia and is considered an important prognostic marker because it may contribute to the high rates of recurrence and/or gastric cancer mortality[9].

Considering the increasing level of understanding of the molecular basis of tumor biology, several biomarkers have been identified for many types of tumors[10]. These biomarkers or molecular markers are molecular entities (DNA, RNA or protein) that can be isolated from biological materials and are useful in the five main areas of cancer study and medicine: cancer screening, diagnosis, tumor classification, prognosis and prediction of a therapeutic response[11]. Despite its importance in translational medicine, some important factors determining the efficiency of a molecular marker assay are the levels of sensitivity and specificity[12], which currently limit their use in clinical practice.

Due to the above-mentioned factors, it is very important to establish molecular markers that can help health professionals in the diagnosis and prognosis of gastric cancer, including those that can be used in a non-invasive way. In this sense, this review aims to present the biomarkers of diagnostic and prognostic use with a broad spectrum of biological samples and detection methods, including genetic, epigenetic and proteomic approaches.

GENETIC MARKERS

Genomic instability

Genomic instability is considered one of the hallmarks of cancer[13]. It can be classified into chromosome instability (CIN) and microsatellite instability (MSI), with the latter being a major pathway involved in gastric carcinogenesis and occurring in at least 20% of all gastric cancer (GC) patients. Several studies have assessed the MSI status of GC patients around the world; however, to date, there are no conclusive studies regarding its significance in the diagnosis and/or prognosis of sporadic or familial gastric cancer[14,15].

MSI usually results from alterations in the genes responsible for DNA repair, such as *MLH1* and *MSH2*, both of which are associated with the development of Lynch syndrome and gastric carcinogenesis[16,17].

In general, the occurrence of MSI in GC is associated with any change (genetic or epigenetic) in DNA repair genes[18]. To define the MSI status of an individual, researchers must assess a panel defined by the National Cancer Institute (BAT25, BAT26, D2S123, D5S346 and D17S250). In this sense, MSI can be considered a prognostic marker, as GC patients who are positive for microsatellite instability (MSI+) have certain features and prognosis, such as tumors located in the antrum and an intestinal phenotype with an expansive growth pattern, especially when associated with *MLH1* methylation[19]. Direct invasion of adjacent organs and extensive nodal metastasis have both been reported, along with a lack of distant metastasis and chemoresistance to fluorouracil treatment[20-22], but with a better prognosis[23], especially in cases of the intestinal type[24,25].

The presence of MSI consequently influences the emergence of mutations in other genes that are important for the maintenance of cellular homeostasis. To date, this association has been reported in genes involved in cell cycle regulation and apoptosis (*TGFβRII, IGFIIR*, *TCF4*, *RIZ*, *BAX*, *CASPASE 5*, *FAS*, *BCL10* and *APAF1*) and in the maintenance of the genomic integrity (*MSH6*, *MSH3*, *MED1*, *RAD50*, *BLM, ATR* and *MRE11*). Consequently, changes in these genes lead to the accumulation of mutations that can result in the development of a malignant phenotype[15,26].

In addition to MSI, another well-studied phenomenon is the CIN phenotype, the most common type of genomic instability observed in solid tumors and a major source of genomic instability in GC. This phenotype is characterized by gross chromosomal abnormalities, such as the gain or loss of entire chromosomes (*i.e.*, aneuploidy) and/or fractions of chromosomes (*i.e.*, loss of heterozygosity, amplifications and translocations)[27-30].

In contrast to MSI, for which the same markers are analyzed in any population, CIN analyzes the entire genome of the individual tumor. In this sense, a common characteristic observed is that several markers may be influenced by the patient's ethnicity. One example is the description of a loss of chromosome 11q observed in diffuse-type GC, which is unique to the population of North Brazil[31].

However, in a broader sense, the results of CIN suggest that several altered chromosome regions are shared independent of the studied population. Therefore, we can observe that losses of chromosome 4q, 9p, 18q, 21q and 22q and gains of chromosomes 5p, 8p, 8q, 17q, 20p and 20q are frequent events in GC and are related to the patient's clinical outcome, as this depends on the amount of DNA copy number alterations[30-33].

It is worth noting that the rearranged chromosomes are always involved with important genes, such those controlling the cell cycle machinery. This was explored by the work of Fan *et al*[32], who used array Comparative Genome Hybridization and found several events of losses

Table 1 Main genetic and epigenetic alterations in gastric cancer tissues and their clinical application as biomarkers

Alteration	Type of alteration	Clinical application	Ref.
HER-2 amplification	Genetic	Prognostic and therapeutic	[34-40]
MYC amplification	Genetic	Progression and metastasis	[52]
TP53 Arg72Pro	Genetic	Risk predictor, prognostic	[63-67,69-72]
CDH1 -160 C>A	Genetic	Risk predictor	[76-78]
CDH1 hypermethylation	Epigenetic	Prognostic, metastasis	[167,170,171]
p16 hypermethylation	Epigenetic	Diagnostic, prognostic and therapeutic	[156-160]

and gains of entire chromosomes and amplifications and deletions of parts of the genome. All of these alterations involved or harbored regions of 321 known or candidate oncogenes (*e.g.*, *MYC*, *HER2*, *TGFB1*), frequently showing copy number gains, and 12 tumor suppressor genes (*e.g.*, *p16*, *SMAD4*, *SMAD7*), showing frequent copy number losses.

Another common feature of gastric tumors is the presence of gene amplification. It is known that the increased production and amplification of *HER2* can be observed in various types of cancer. Several clinical studies have been able to identify HER2 protein overexpression or *HER2* gene amplification in gastric cancer, with great variation in the number of patients with HER2-positive tumors[34]. Although the prognostic value of this biological marker remains questionable for resected gastric cancer[35-37], it is well documented that this amplification event is more frequent in intestinal-type GC[38-40] and is significantly associated with a poor prognosis[34-40]. Furthermore, this gene amplification is considered an important biological marker for guiding clinical decisions regarding adjuvant chemotherapy with trastuzumab, especially in patients with lymph node metastasis, as it predicts sensitivity to this chemotherapeutic agent[34,36,38,41-45].

The overexpression of the *MYC* gene, especially due to gene amplification, was described as a frequent event in GC, ranging from 15.6% to 100% in primary tumors, especially those of the intestinal type[46-51]. In a recent study, de Souza *et al*[52] demonstrated the overexpression of MYC in gastric tumors, linking it to tumor progression (deeper tumor extension and the presence of distant metastasis).

Mutations and polymorphisms

As genetic alterations have a clear influence on the development and outcome of cancer treatment, it is expected that gene-based markers have a significant impact on tumor control. Among the most prevalent and common genetic alterations in GC are mutations in the *TP53* and *CDH1* genes (Table 1). However, in terms of biomarkers of diagnosis and prognosis, there is some divergence in the results with respect to the occurrence of mutations and their relationship to the histological characteristics of the tumor or stage in GC[53-55].

In addition to mutations, other important genetic alterations influencing gastric tumorigenesis are single-nucleotide polymorphisms (SNPs), which are responsible for over 90% of the variation in the human genome[56]. It is known that infections and nutritional, environmental and genetic factors have a direct link with gastric carcinogenesis. However, individuals exposed to these factors that actually develop gastric cancer belong to a small group, suggesting that the genetic susceptibility, mainly SNPs, of the host must be taken into consideration[57-59].

The number of studies linking genetic polymorphisms and GC has increased exponentially over the past decades, in parallel with major advances in sequencing and genotyping, and polymorphisms may be useful indicators for assessing the risk of gastric cancer[60]. However, it is worth noting that the results derived from polymorphism studies still need to be carefully interpreted, as these biomarkers are generally population dependent, with a strong ethnic influence.

One well-studied polymorphism is *TP53* Arg72Pro, which remains controversial with regard to its potential as biomarker. Although no association with GC risk was observed in Turkish[61] and Korean[62] populations, several meta-analyses indicate its potential use as a risk predictor for Asian but not Caucasian populations[63-67]. According to Francisco *et al*[68], this difference must be related to ethnicity, as it may modulate the penetrance of Arg72Pro in cancer susceptibility.

In addition to its application as a risk predictor, this polymorphism has recently been used as a prognostic factor because it may be correlated with the clinical outcome of patients receiving chemotherapy, though with contradictory results. Wang *et al*[69] observed that the Arg allele is related to an unfavorable effect on patients treated with 5-fluorouracil (5-FU). However, different results were obtained by several other works in which the Pro allele was related to poor survival in patients using 5-FU[70], oxaliplatin[71] or a combination of paclitaxel and cisplatin[72]. Therefore, although promising, the use of the Arg72Pro polymorphism in this sense should be carefully analyzed.

Another studied polymorphism is -160C>A, which is located in the promoter region of *CDH1*, a gene that encodes a transmembrane glycoprotein responsible for mediating intercellular adhesion and cell polarity and plays an important role not only in the regulation of morphogenesis of normal and neoplastic tissues but also in tumor invasion and metastasis[73].

It has been described that the A allele of this polymorphism results in an approximately 68% reduction in transcriptional activity in comparison to the C allele[74,75] and has been associated a with the negative regulation of *CDH1*, which can lead to the loss of cell-to-cell adhesion

mediated by E-cadherin, resulting in increased susceptibility to tumor development and subsequent tumor invasion and metastasis[76]. Thus, the variant allele was suggested to be a likely genetic marker for an increased risk of GC[77,78].

A considerable number of studies have been conducted to investigate the association between this polymorphism and susceptibility to GC in humans, with conflicting results, which may also be explained by the ethnic composition of each population studied[55,60,79-84].

Although the AA genotype is related to an increased risk of GC in the Oman population[74] and Caucasians[73,76], several meta-analyses did not find any influence on the overall risk for the studied populations (Caucasians, Asians and mixed). However, when stratified by ethnicity, the results suggest a protective effect of the A allele in Asian populations[75,85,86].

Two other genotypes in *CDH1*, 347G>GA and +54T>C, were significantly associated with the risk of GC in a study conducted in China[87]. However, two studies in Japan[88] and Italy[89] did not confirm this relationship. According to Pan *et al*[90], to reach a definitive conclusion, further studies with better designs are needed to explore the association of *CDH1* gene polymorphisms with GC susceptibility.

PROTEOMICS

Proteomic-based techniques in cancer biology, such as 2-DE (two-dimensional electrophoresis), iTRAQ (isobaric tags for relative and absolute quantitation), ICAT (isotope-coded affinity tag), protein chip array and liquid chromatography, have been used to identify and quantify proteins that can be used as biomarkers in bodily fluids and tissues in GC[91].

Human serum contains a complex array of peptides. Some of these may function as biomarkers, with their presence/absence or relative abundance being correlated with health status and thus useful for prognosis or diagnosis[92]. To date, the most common fluid biomarkers available for GC include carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA 19-9), carbohydrate antigen 72-4 (CA 72-4), Cytokeratins (CYFRA 21-1, TPA - tissue polypeptide antigen, TPS - tissue polypeptide-specific antigen), E-cadherin, pepsinogen, cytokines and the β-subunit of HCG. However, although some authors suggest that the sensitivity and specificity of these markers are not sufficient for the diagnosis of GC[93,94], their use in clinical practice is recommended by most authors because they are useful as prognostic, diagnostic and peritoneal recurrence markers[95-100]. The use of CEA and CA 19-9 as prognosis markers, for example, is recommended because their levels increase according to the tumor stage; these markers are especially useful when a cutoff ratio (divided in three stages: negative, low and high) is applied[94-96].

An expansive bibliography about new biomarkers in biological fluids of GC has accumulated over time[101-106]. These biomarkers include, for example, tubulin beta chain, thymosin beta-4-like protein 3, cytochrome b-c1 complex subunit 1, aromatic amino acids (tyrosine, phenylalanine, tryptophan), S100A9/AAT and S100A9/GIF, collagen type IV, hyaluronic acid, prostaglandin E2, EGF, TGFα, epidermal growth factor receptor (EGFR), proapolipoprotein A1 (proApoA1), apolipoprotein A1, transthyretin (TTR), D-dimer, vitronectin (VN), interleukin-6, a-2 macroglobulin, C-reactive protein and plasminogen activator inhibitor-1[107]. However, most of these biomarker candidates still need to be extensively validated in large clinical cohorts because they have been identified in many studies with different methods over time.

It is worth mentioning that other sources of proteins, such as tissue samples and cell lines, have been used in the discovery of new GC biomarkers. To date, tissue samples have not been widely used for this purpose due to poor reproducibility and the small overlap between studies as well as conflicting data. Moreover, in most of these studies, etiological differences between diffuse and intestinal tumor subtypes were ignored because finer sample classification was not possible with the limited patient materials[107]. Due to these difficulties, modern techniques in proteomic studies have enabled a much higher number of proteins in GC tissues to be described, including selenium-binding protein 1, ENO1, ADHIC, ETFB, VDAC, DMBT1, LTF, GRP78, GRP94, PPIA, PRDX1, PTEN, CRIP1, HNP-1, S100A6, S100A8, S100A9, α-defensin-1 and α-defensin-2[107-111]. Other proposed candidate biomarkers include CRIP1, HNP-1, and S100-A6[112] and human neutrophil peptides 1-3 (HNPs 1-3) and MIF[113]. In summary, the detection and verification of tissue biomarkers through the application of various proteomic methods can promote the more robust clinical evaluation of patients with gastric cancer.

As reported, the majority of tumor biomarkers in GC diagnosis are glycoproteins[114], with the most common being mucin-5AC (MUC5AC), IgG, mucin-1 (MUC1), IGHM, LRG1, haptoglobin (HP), albumin (ALB), TF, kininogen-1 (KNG1), alpha-1-acid glycoprotein (AGP), ceruloplasmin (CP), A1BG, vitamin D binding protein (GC), alpha-1-antitrypsin (SERPINA1), antithrombin (SERPINC1), angiotensin (AGT), CFB, serpin peptidase inhibitor, Clade A (SERPINA3), alpha-2-HS-glycoprotein (AHSG), Zn-alpha-2-glycoprotein (AZGP1), CLU, ITIH2, complement factor H (CFH), interalpha-trypsin inhibitor HCRP, SERPING1 and C4A variant protein (C4A)[115-118].

Recently, Li *et al*[119] studied two multidrug-resistant cell lines and their parental drug-sensitive GC cell line to characterize the multiple drug resistance (MDR)-related cell surface glycoproteome. These authors successfully identified 56 cell membrane glycoproteins, 11 of which (Mesothelin, EGFR, Integrin alpha-3, CD59, Folate receptor alpha, Peptidyl-prolyl cis-trans isomerase FKBP9, Laminin subunit alpha-5, Dihydropyridine receptor alpha 2, Multidrug resistance protein 1, Prostaglandin F2 receptor negative regulator and Golgi apparatus protein 1) were

Table 2 miRNAs differently expressed in gastric cancer tissues and their clinical application

miRNA	Level of expression	Clinical application	Ref.
miR-301a	Up-regulated	Progression and prognostic	[127]
miR-29 family	Down-regulated	Prognostic and therapeutic	[128]
miR-146a	Down-regulated	Metastasis	[129]
miR-10b	Up-regulated	Progression and prognostic	[130]
miR-107	Up-regulated	Prognostic	[131]
miR-345 + miR-142	Up-regulated (miR-345) and Down-regulated (miR-142)	Recurrence and progression	[132]
let-7i	Down-regulated	Prognostic and therapeutic	[133]
miR-221	Up-regulated	Progression, prognostic and therapeutic	[125,134]
miR-148a	Down-regulated	Prognostic	[135]
miR-155	Down-regulated	Progression and metastasis	[136]
miR-129-2-3p	Down-regulated	Progression	[137]
miR-181b	Up-regulated	Prognostic	[138]
miR-21	Up-regulated	Prognostic	[138,139]

found to be differentially expressed with the same trend in both the drug-resistant and sensitive cell lines. This report was the first concerning the relationship between glycoprotein alterations and MDR in gastric tumors and was also helpful for better interpreting the sophisticated mechanisms of MDR in gastric cancer, which, of course, still require further investigation and verification.

Given the current multiplicity of proteomic studies in GC, due to the vast amounts of data generated, it is important to maintain an up-to-date and searchable index of the lists of biomarkers obtained in different studies. Finally, it is essential that future studies focus not only on identifying the disease-associated alterations in proteins but also on determining the cellular functions of the proteins identified as well as the mechanistic networks in which they participate. The biomarkers identified experimentally should serve as entry points for investigating the mechanisms of carcinogenesis and tumor progression.

EPIGENETICS

MicroRNA

MicroRNAs (miRNAs) are small (typically about 22 nt in size) regulatory RNA molecules that modulate the activity of specific mRNA targets and play important roles in a wide range of physiologic and pathologic process. miRNAs generally disrupt gene expression by inhibiting translation or through the cleavage of the target mRNA[120]. When associated with the tumor process, miRNAs are called oncomiRs; they may act as oncogenes or as tumor suppressor genes. As a result, oncomiRs can be used in the diagnosis and treatment of cancer, as the expression patterns of miRNAs in human cancer appear to be tissue specific[121]. In addition, genome-wide studies have shown that miRNA genes are frequently located within regions of heterozygosity loss and amplification and fragile sites, suggesting the vital role of miRNAs in tumorigenesis[122].

miRNAs have shown great potential as tissue-based markers for cancer definition. The presence of a miRNA signature in gastric cancer has been suggested by some authors, with specific genes being up- and down-regulated, which can be useful in the diagnostic process[123-125]. Moreover, due to their size, abundance, tissue specificity and relative stability in the circulation of biological fluids, these molecules can serve as accessible biomarkers to detect and monitor GC[126] (Tables 2 and 3).

Recently, miRNA studies have focused on the prediction of chemotherapeutic resistance, as some of those molecules, such as miR-19a/b and miR-106a, accelerate drug efflux, acting as a barrier to the success of GC chemotherapy[146,147].

Methylation and histone modifications

DNA methylation is an epigenetic modification in both prokaryotes and eukaryotes and occurs at carbon 5 of the cytosine ring within CpG dinucleotides, especially in the promoter region of several genes and in noncoding genomic regions[148,149]. Because DNA methylation has a tissue-specific pattern, is involved in a variety of cellular processes, such as gene expression regulation, genomic imprinting, transcriptional regulation and cellular differentiation, and can be modified during tumorigenesis, it is used as a molecular marker of the tumor-development process[150-152].

In GC patients, it is suggested that the methylation pattern of some genes is dependent on environmental factors, such as the presence of *H. pylori*[153-155], as well as on the patient's age[153]. Therefore, biomarkers should be carefully selected to avoid false results in a prognostic and diagnostic approach.

An aberrant methylation pattern of several genes is currently associated with GC (Table 1). One of these genes is the classical tumor suppressor gene *p16*, which was identified as a diagnostic[156,157] and prognostic biomarker in several populations because it can be related to better survival in patients who received 5-fluoracil therapy[158], to metastasis and poor survival in patients without neoadjuvant therapy[159] and to tumor location[160].

Several other genes with altered methylation patterns were identified as potentially useful prognostic biomarkers, including *RKIP*[161], *ADAMTS9*[154], *XAF1*[162], *BCL6B*[163], miR34b and miR129-2[164] and *HOXD10*[165], but studies have only been performed in a few Asian

Table 3 miRNAs differently expressed in body fluids from gastric cancer patients and their clinical application

miRNA	Body fluid	Level of expression	Clinical application	Ref.
miR-200c	Blood	Up-regulated	Progression and survival	[140]
miR-421	Gastric Juice	Up-regulated	Screening	[141]
miR-21	Gastric Juice	Up-regulated	Screening	[142]
miR-106a	Gastric Juice	Up-regulated	Screening	[142]
miR-129-1-3p	Gastric Juice	Down-regulated	Screening	[137]
miR-129-2-3p	Gastric Juice	Down-regulated	Screening	[137]
miR-335	Blood	Up-regulated	Recurrence and prognostic	[143]
miR-221	Serum	Up-regulated	Screening	[144]
miR-744	Serum	Up-regulated	Screening	[144]
miR-376c	Serum	Up-regulated	Screening	[144]
miR-199a-3p	Plasma	Up-regulated	Progression, screening	[145]

populations.

Some of the markers analyzed to date have methylation patterns that are related to the patient's chemosensitivity, such as *MGMT*, *MLH1*, *BNIP3*, *DAPK* and *BMP4*[166-168]. As a result, these genes may be useful for predicting the best treatment for each patient.

One of the most interesting features of methylation markers is the fact that many of them may be used as non-invasive makers, as they can be detected in body fluids such as serum, plasma and peritoneal wash.

One of the most commonly used markers in body fluids is the *CDH1* gene methylation pattern, the main mechanism responsible for *CDH1* down-regulation[169]. The altered methylation pattern of this gene may be detected in peritoneal fluid and used as a marker of tumor recurrence, metastasis and tumor stage[167,170] or in serum, where it is used together with the *APC* methylation status as a marker of prognosis[171].

Some other markers may be detected in serum, such as *SFRP2*[172] and *SLC19A3*[173], or gastric washes, such as a combination of *MINT25*, *ADAM23* and *GDNF*[174]; these are useful as diagnostic markers.

Although studies associating the methylation status of a particular gene and tumorigenesis are frequent, those associating histone modifications, as well as the enzymes responsible, are still few. The majority of such studies are related to histone deacetylase enzymes, which are considered molecular markers of prognosis, with the expression of HDAC 1 and 2 being related to tumor aggressiveness[175,176].

Concluding remarks

Advances in technology have allowed the development of several methods to understand the mechanisms underlying gastric carcinogenesis, resulting in the identification of a large number of molecular targets that can be used as biomarkers with diagnostic and prognostic potential. Several of these (especially *HER-2* amplification, miR-19a/b, miR-160a and *p16* hypermethylation) can also be used for the prediction of therapeutic response, which is a tremendous help to clinicians. Despite this, many of these biomarkers, especially genetic markers, have been tested in only one or a few populations. We must consider that GC, as with other types of tumor, is influenced by ethnic and environmental factors, which can result in the following question: how universal can a prognostic/diagnostic genetic marker be? Thus far, there is unfortunately no answer to that question, and we believe that it will be a long time until this question may be conclusively answered. Therefore, the simplest approach at present is to validate the discovered markers in the target population and to use several biomarkers for each patient. One alternative could be the use of a proteomic approach, which only analyzes protein expression and is independent of the cause (genetic or epigenetic) of any altered pattern. However, there are some limitations to that approach, such as the availability of studies in only a few populations and the cost of the analysis, which remains very high.

Conversely, epigenetic markers appear to be much more tumor specific, as their pattern has been confirmed in all of the studied populations. Moreover, epigenetic markers are more prone to become target markers for therapeutics trials, as these types of alterations are reversible.

Therefore, one might carefully select molecular markers depending on their use. We must bear in mind that genetic markers are much more dependent on the ethnic component than epigenetic markers, making the latter a currently much more reliable option for clinicians.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (8): Gastric cancer

Towards personalized perioperative treatment for advanced gastric cancer

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Abstract

Gastric cancer is one of the most frequently diagnosed cancers worldwide. Although the rate of gastric cancer has declined dramatically over the past decades in most developed Western countries, it has not declined in East Asia. Currently, a radical gastrectomy is still the only curative treatment for gastric cancer. Over the last twenty years, however, surgery alone has been replaced by a multimodal perioperative approach. To achieve the maximum benefit from the perioperative treatment, a thorough evaluation of the tumor must first be performed. A complete assessment of gastric cancer is divided into two parts: staging and histology. According to the stage and histology of the cancer, perioperative chemotherapy or radiochemotherapy can be implemented, and perioperative targeted therapies such as trastuzumab may also play a role in this field. However, perioperative treatment approaches have not been widely accepted until a series of clinical trials were performed to evaluate the value of perioperative treatment. Although multimodal perioperative treatment has been widely applied in clinical practice, personalization of perioperative treatment represents the next stage in the treatment of gastric cancer. Genomic-guided treatment and efficacy prediction using molecular biomarkers in perioperative treatment are of great importance in the evolution of treatment and may become an ideal treatment method.

Key words: Gastric cancer; Pre-therapeutic evaluation; Perioperative chemotherapy; Perioperative radiochemotherapy; Perioperative target therapy

Core tip: Multimodal perioperative treatment of advanced gastric cancer is playing an increasingly important role in patient treatment. Different strategies, including preoperative and postoperative chemotherapy and radiochemotherapy, are implemented in clinical practice and a new concept of perioperative-targeted therapy is emerging. Although many randomized clinical trials have been performed to determine the effectiveness of these therapies over surgery alone, little evidence exists regarding the comparison of the different therapies. Personalized treatment should be based on the results of randomized clinical trials as well as subgroup analyses, tailored by histology, demography, and predictors, including tumor markers and genomic profiling.

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INTRODUCTION

Gastric cancer is one of the most frequently diagnosed cancers around the world. Until the mid-1990s, it was the first cause of cancer death in the world and now it represents the fourth most common cancer[1,2]. While gastric cancer is the 14th most common cancer in the United States, it is the 2nd most common cancer in China[3,4]. Although the effects of geography on the incidence and prognosis are still not clearly understood, factors of gastric carcinogenesis, diagnosis, and therapeutic strategies may contribute to the differences[5].

Radical gastrectomy, the complete surgical resection of macroscopic and microscopic tumors (R0 resection), is still currently the only way to cure gastric cancer. The extent of lymphadenectomy, however, has been controversial between the East and West until recent years. Radical gastrectomy with extended D2 lymphadenectomy is considered the standard surgical practice in East Asia and has been accepted in the West. Nevertheless, limited D1 resection with radiochemotherapy is still more frequently implemented in Western countries[5-7].

In contrast to those with early gastric cancers (EGCs), patients diagnosed with advanced gastric cancers (AGCs) typically have a poor prognosis. According to the 7th edition of the American Joint Committee on Cancer (AJCC) tumor-node-metastasis (TNM) staging of gastric cancer, the 5-year survival rate of patients with AGCs was 9.2%-45.5% in the United States[8], and 40%-60% of patients with local AGCs experience recurrence after surgery[9]. Over the last few decades, surgery as the sole form of treatment has been replaced by different forms of multimodal treatment of AGCs around the world[10-12]. To achieve the most benefits from the perioperative treatment of AGCs, a thorough evaluation of the tumor is required. Different stages and histological types of gastric cancers have different biological behaviors and thus respond differentially to treatment, a factor that hits at the core of personalized perioperative treatment for gastric cancer.

This article will review the current strategies towards personalized perioperative treatment of gastric cancer and discuss the appropriate indications for perioperative treatment, multidisciplinary approaches for AGCs, as well as the future questions that remain in the tailored management of gastric cancer.

PRETHERAPEUTIC EVALUATION OF AGC

According to the National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology of Gastric Cancer and other guidelines worldwide, a complete endoscopic evaluation of the esophagus, stomach, and duodenum with a biopsy of any suspicious lesion is considered the gold standard for detection and histological verification of gastric cancer[11,13,14]. Because the number and location of the biopsies are still controversial and limited evidence-based data exist to address the controversy, a variety of recommendations in national guidelines have been suggested. Aside from verifying malignant disease histologically, the goal of biopsies is to evaluate the histological tumor type and examine the biological behavior of the tumor, if possible, by appropriate sampling. Different types of gastric cancer may respond differentially to chemotherapy or radiotherapy. For example, hepatoid adenocarcinoma, a rare form of gastric cancer, responds poorly to chemotherapy and the best strategy is to operate as early as possible[15]. However, several different histological classifications of gastric cancer currently exist and include the Lauren classification, Japanese Gastric Cancer Association classification, and World Health Organization (WHO) classification[16,17]. The lack of consensus in histological classification reveals an insufficient understanding of the biological behavior of gastric cancer. Inconsistencies between the biopsy and postoperative histology occur frequently, which limits the implementation of personalized treatment by histology alone. Therefore, TNM stage-oriented treatment is the gold standard for preoperative treatment of gastric cancer. The heterogeneity of gastric cancer greatly contributes to the personalized treatment and represents one of the main challenges in perioperative treatment.

Evaluation of the tumor infiltration stage (T-stage) is the main parameter to distinguish AGCs from EGCs. The current gold standard for T-staging is endoscopic ultrasound (EUS), which has an accuracy between 65% and 92%[18] and a sensitivity and specificity of 88% and 100% for T1, 82% and 96% for T2, 90% and 95% for T3, and 99% and 97% for T4, respectively[19]. Multi-detector computed tomography (MDCT) for T-staging is less accurate than EUS, though the sensitivity and specificity of serosa involvement are similar to EUS[18,19]. A meta-analysis involving nine studies utilizing positron emission tomography (PET) to evaluate gastric cancer reported that, despite the inability to stage gastric cancer by tumor depth, PET has a pooled primary tumor detection ratio of 80% in identifying the existence of gastric cancer[20].

Lymph node involvement (N-stage) represents the greatest challenge in gastric cancer staging. N-staging is currently achieved by evaluating the number of metastatic lymph nodes according to the 7th AJCC TNM staging system[8]. Currently, lymph node size is the primary parameter used to define nodal involvement. Micrometastasis without lymph node enlargement is not detected by imaging methods such as MDCT or EUS. The sensitivity and specificity for N-staging with EUS is approximately 50%-60% and 85%-95%[19], respectively, and MDCT is not superior to EUS[18,19]. PET can evaluate node metabolism using the standardized uptake value (SUV) in addition to acquiring the size of the lymph nodes. However, the mean SUV noted for N-staging can also vary, with overall values ranging from 4.5 to 6.8, and an overall accuracy of 17.7% to 79.2%[20].

Distant metastasis (M-stage) is predominantly evaluated with thoracic, abdominal, and pelvic MDCT with a sensitivity and specificity of > 70% if performed using a biphasic protocol (including a portal venous contrast phase) and a slide thickness < 3 mm[14,21]. Thus, MDCT is

considered the gold standard approach for assaying solid organ metastasis. PET is also one of the best methods to assess the M-stage of gastric cancer with an overall accuracy of 88%[20], but studies comparing PET with MDCT for M-staging are still lacking[20]. Because of the high prevalence of peritoneal carcinomatosis, additional attention must be paid to T3 and T4 patients[22-24]. Laparoscopic exploration should be employed to exclude liver metastasis and peritoneal carcinomatosis. Detection of free cancer cells by peritoneal lavage cytology can predict the risk of peritoneal carcinomatosis with high specificity and this patient category may also benefit from hyperthermic intraperitoneal chemotherapy (HIPEC), which has been shown to improve overall survival and decrease peritoneal local recurrence[25]. Metabolic imaging represents another advantage of PET in evaluating gastric cancer, as it may provide clues to predict treatment responses, which will be discussed later in this article.

MOLECULAR AND RADIOLOGIC ASPECTS OF PERSONALIZED PERIOPERATIVE TREATMENT

Researchers worldwide have been working to identify the molecular subtypes of gastric cancer and their differential responses to chemotherapy. Lei and colleagues identified three subtypes of gastric adenocarcinoma: proliferative, metabolic, and mesenchymal. In the study from Lei *et al*[26], cancer cells from the metabolic subtype were more sensitive to and reaped greater benefits from 5-fluorouracil (5-FU) than the other subtypes. Meanwhile, tumors of the mesenchymal subtype contained cells with features of cancer stem cells, and cell lines of this subtype were particularly sensitive to phosphatidylinositol 3-kinase-AKT-mTOR inhibitors *in vitro*. This study has been touted by some experts in the field as a new direction for personalized therapy of gastric adenocarcinoma and they are finding ways to apply this information to identify tumor subsets and develop molecularly tailored, individualized therapies[27]. Although many other studies have been conducted, there is still no consensus on the molecular subtypes of gastric cancer[28-31]. Recently, several studies have focused on predicting the efficacy of chemotherapy using genome-guided chemotherapy. Molecular biomarkers including VEGFR-1 and ERCC1/TS mRNA levels[32] were reported in the 2013 International Gastric Cancer Congress[33]. While there is still a long way before these studies can be translated into clinical practice, clinical trials may provide some clues for the choice of treatment regimen in the postoperative setting.

Diffusion-weighted MRI (DW-MRI) is a promising imaging technique to evaluate cancer treatment response, as it is sensitive enough to detect the macromolecular and microstructural changes that occur at the cellular level prior to anatomical changes during therapy[34]. Studies have shown that successful treatment of many tumor types can be detected using DW-MRI to measure the early increase in the apparent diffusion coefficient (ADC) values[35-38]. Additionally, a low pretreatment ADC value is often predictive of a better outcome[34], which may provide an important opportunity for individualized therapy, minimizing unnecessary toxicity associated with ineffective therapies and improving overall patient health care at a lower cost. The efficacy of DW-MRI in gastric cancer, however, has only been evaluated in a few cases[39,40].

Because of the nature of metabolic imaging, PET can provide information on the metabolic response of gastric cancers. A series of studies have been performed to assess the utility of PET in predicting the response to gastric cancer treatment[41-49]. In these studies, a metabolic response was defined as a decrease of $\geqslant$ 35% in the tumor glucose SUV after preoperative chemotherapy, which can be predicted by fluorodeoxyglucose PET. These studies suggested that the metabolic response may correlate with tumor response, ultimately translating into improved patient survival[41-48].

PERIOPERATIVE CHEMOTHERAPY

Over the past decades, gastric cancer treatment by surgery alone has been replaced by a multimodal treatment approach consisting of surgery and pre- or postoperative chemotherapy or radiochemotherapy. In addition to the wide clinical application of multimodal treatment, personalized perioperative treatment represents the future of gastric cancer treatment.

Postoperative chemotherapy

The survival benefits of postoperative chemotherapy differ in clinical trials between Eastern and Western countries. In 1993, Hermans and colleagues performed a meta-analysis on 11 clinical trials from 1980 and found that postoperative chemotherapy for resectable gastric cancer did not, in general, improve survival[49]. In contrast, the Global Advanced/Adjuvant Stomach Tumor Research International Collaboration Group conducted a meta-analysis on 17 randomized clinical trials (RCTs) including 3838 patients with resectable gastric cancer and reported that postoperative chemotherapy was associated with a statistically significant benefit in terms of overall survival (HR = 0.82, 95%CI: 0.76-0.90; $P < 0.001$) and disease-free survival (HR = 0.82, 95%CI: 0.75-0.90; $P < 0.001$)[50]. This meta-analysis supports the utility of postoperative chemotherapy in resectable gastric cancer.

In contrast to the small benefit observed on overall survival in the Western clinical trials, favorable outcomes were observed in RCTs in the East. A large RCT from the Adjuvant Chemotherapy Trial of S-1 for Gastric Cancer (ACTS-GC) in Japan randomly assigned 1059 patients with stage Ⅱ or Ⅲ gastric cancer who underwent gastrectomy with extended (D2) lymph node dissection to groups with or without S-1 adjuvant chemotherapy[51]. In this study, the 3-year overall survival rate was 80.1% in the S-1 group and 70.1% in the surgery-only group (HR = 0.68, 95%CI: 0.52-0.87; $P = 0.003$). However, the high

overall survival rate at 3 years in both groups has not been replicated in any Western trials. The United States multicenter phase Ⅲ study comparing cisplatin/S-1 with cisplatin/infusional fluorouracil in advanced gastric or gastroesophageal adenocarcinoma stratified more than 1000 patients to compare the overall survival between the postoperative chemotherapy regimens of cisplatin/S-1 and cisplatin/5-FU but failed to confirm the results from the ACTS-GC trial and showed that cisplatin/S-1 did not prolong overall survival of patients with advanced gastric or gastroesophageal adenocarcinoma when compared with cisplatin/5-FU[52].

In addition to S-1, capecitabine, another form of oral fluoropyrimidine, with oxaliplatin (XELOX) was evaluated in the Capecitabine and Oxaliplatin Adjuvant Study in Stomach Cancer (CLASSIC) study, which was a multicenter, randomized, phase Ⅲ trial occurring across 37 centers (n = 1035 patients) in South Korea, China, and Taiwan[53]. The 3-year disease-free survival was 74% in the chemotherapy and surgery group in comparison to 59% in the surgery only group (HR = 0.56, 95%CI: 0.44-0.72; $P < 0.0001$). A recent update of the CLASSIC trial has also been reported. After a median follow-up of 5 years, a 34% reduction in the risk of death with chemotherapy versus surgery alone was observed (HR = 0.66, 95%CI: 0.51-0.85; $P = 0.0015$)[54]. The 5-year overall survival rates were 78% in the XELOX group and 69% in the surgery alone group ($P = 0.0029$). This update further proved the efficacy of XELOX as a treatment regimen for postoperative chemotherapy.

A meta-analysis from Janunger *et al*[55] also found that there was a significant difference in the effect of chemotherapy on AGCs between Asian and European patients. This study has raised the issue about whether there are ethnic differences between gastric cancer patients in the East and West. RCT results should always be tracked back to the population from whom the study group was sampled, which is an important principle of personalized medicine.

The results of the above clinical trials and meta-analyses indicate that 5-FU (or its derivatives) postoperative chemotherapy may bring selected patients with resectable gastric cancer a higher probability of survival, but the studies are insufficient at predicting how individuals will respond. Thus, there is still a long way to go towards achieving personalized postoperative chemotherapy.

The subgroup analysis of the ACTS-GC and CLASSIC trials highlights the future direction of personalized postoperative chemotherapy. In the ACTS-GC trial, male patients < 60 years of age with stage Ⅱ (6th TNM classification), stage T2 (tumor invades the muscularis propria or the subserosal connective tissue), stage N1 (1-6), and undifferentiated histological tumors may benefit most from postoperative S-1 chemotherapy, although it is important to note that the difference was not statistically significant. In a similar analysis performed in the CLASSIC trial, male patients < 65 or ≥ 65 years of age with stage Ⅱ (6th TNM classification) and stage N1 or N2 (1-15 involved nodes) respond more favorably to postoperative XELOX chemotherapy when 3-year disease-free survival was examined. A comparison between the two treatment regimens suggests that patients older than 65 years of age or with lymph node metastasis in 7-15 nodes may benefit more from the XELOX regimen than S-1. This type of comparison between different trials, however, does not provide solid evidence of one treatment having an advantage over another; RCTs are still required to provide additional evidence for personalized perioperative chemotherapy.

What is the best chemotherapy regimen and course of treatment? Dozens of clinical trials including the NCT01426646, NCT00343668, and NCT01531452 are currently being performed to address this question. It seems be an answerless question with the increasing number of cytotoxic drugs being developed. Studies must also find a way to predict the effects of different treatments. Moreover, there is currently no way to assess how these treatments affect individuals, rather than populations.

Preoperative chemotherapy

Preoperative chemotherapy is commonly applied in Europe and this clinical practice is based on the results of three major RCTs. The Medical Research Council Adjuvant Gastric Infusional Chemotherapy (MAGIC) trial, a British multicenter RCT, randomly assigned over 500 patients with histologically verified adenocarcinoma of the stomach or gastroesophageal junction to either surgery alone or surgery following chemotherapy with epirubicin, cisplatin, and 5-FU[56]. With a median follow-up of four years, the preoperative chemotherapy group had a higher likelihood of overall survival (HR = 0.75, 95%CI: 0.60-0.93; $P = 0.009$; 5-year survival rate: 36% *vs* 23%) and of progression-free survival (HR = 0.66, 95%CI: 0.53-0.81; $P < 0.001$). This trial was limited by the heterogeneous inclusion criteria, which included patients with gastric cancer, gastroesophageal cancers, and cancers of the distal esophagus, as well as the lack of quality control of surgical and pathological operations. Moreover, more than half of the patients in the preoperative group did not complete the chemotherapy regimen, making it difficult to evaluate the effects of preoperative chemotherapy from postoperative chemotherapy. Thus, it is important to remember that the results of the MAGIC trial are not sufficient to confirm the effects of preoperative chemotherapy on gastric cancer following curative gastrectomy with D2 lymphadenectomy.

The ACCORD07/FFCD-9703 French trial obtained similar results to the MAGIC trial[57]. Two hundred and twenty-four patients with resectable cancer of the lower esophagus, gastroesophageal junction, or stomach were enrolled to either a surgery alone group or to the preoperative chemotherapy group, which received two or three preoperative cycles of intravenous cisplatin and a continuous intravenous infusion of 5-FU for five consecutive days every 28 d and three or four postoperative cycles of the same regimen in addition to the surgery.

Compared with the surgery alone group, the preoperative chemotherapy group had better overall (38% *vs* 24%; HR = 0.69, 95%CI: 0.50-0.95; $P = 0.02$) and disease-free (34% *vs* 19%; HR = 0.65, 95%CI: 0.48-0.89; $P = 0.003$) 5-year survival rates. In a multivariate analysis, preoperative chemotherapy ($P = 0.01$) and stomach tumor localization ($P < 0.01$) were favorable prognostic factors for survival, and preoperative chemotherapy significantly improved the curative resection rate (84% *vs* 73%, $P = 0.04$). The same limitation of heterogeneous inclusion criteria still exists in this trial and does not answer the remaining questions from the MAGIC trial.

To address the questions remaining in the trials mentioned above, the EORTC 40954 trial was performed[58]. This trial used stringent inclusion criteria to include only locally advanced adenocarcinoma of the stomach and gastroesophageal junction (SIEWERT Ⅱ and Ⅲ). Patients in this trial were randomly assigned to either undergo surgery alone or receive surgery in combination with preoperative chemotherapy consisting of cisplatin, 5-FU, and leucovorin. In contrast to the above trials, rigorous preoperative staging and quality control of surgery were applied in this trial. However, this trial was halted due to poor accrual after 144 patients were assigned. Out of the 144 included patients, 52.8% had tumors located in the proximal third of the stomach (including SIEWERT Ⅱ and Ⅲ) and the R0 resection rate was 81.9% after preoperative chemotherapy, as compared with 66.7% with surgery alone ($P = 0.036$). After a median follow-up period of 4.4 years and 67 deaths, a survival benefit could not be shown (HR = 0.84, 95%CI: 0.52-1.35; $P = 0.466$). An overall survival of 64.6 mo was observed in the chemotherapy group in comparison to 52.5 mo in the surgery alone group. This trial showed a significantly increased R0 resection rate, but failed to demonstrate a survival benefit. Possible explanations of why a survival benefit was not observed in the study include a low statistical power, a high rate of proximal gastric cancer and a better outcome than expected after radical surgery alone due to the high quality of surgery, which included resections of regional lymph nodes outside the perigastric area. Another limitation that needs to be considered is the possibility of increased morbidity and mortality of the operation after preoperative chemotherapy.

The differing results from these three trials might be associated with the quality control of the surgery. Only the EORTC 40954 trial applied the D2 lymphadenectomy and may render the negative effects of preoperative chemotherapy more apparent. The specific treatment regimen used for preoperative chemotherapy is another caveat that must be addressed. The regimens used in the three trials above are not recommended in East Asia, despite being included in the NCCN and European Society for Medical Oncology guidelines[10-12]. Clinical trials to compare different regimens have been performed worldwide. There are currently more than 50 clinical trials registered on the clinicaltrials.gov website, with a broad spectrum of regimens and drugs.

Proper imaging evaluation should be performed at the appropriate time to achieve the utmost benefit prior to surgery. Currently, the Response Evaluation Criteria In Solid Tumors 1.1 criteria, WHO criteria, and many other sets of criteria are used to evaluate the effectiveness of preoperative treatment[59], but limitations still exist. None of these criteria can accurately predict a gastric cancer patient's response to chemotherapy. New imaging methods like diffusion weighted imaging and PET may provide clues for this problem. As to the pathology evaluation, the widely applied Tumor Regression Grade was developed from patients with rectal cancer and is still not specific enough for gastric cancer. Patients diagnosed as M1 may benefit from preoperative chemotherapy due to the free cancer cells in a peritoneal lavage cytology examination. These patients received HIPEC together with preoperative chemotherapy, which converted some patients to ypM0 when a second peritoneal lavage cytology examination was performed, giving them a potential R0 operation[24]. This modality is now being evaluated in our center (NCT01471132).

PERIOPERATIVE RADIOCHEMOTHERAPY

Although postoperative radiochemotherapy is currently used in the United States as a standard treatment of AGCs, it is not widely accepted in other parts of the world. In 1984, a small prospective clinical trial that included 60 patients evaluated the value of postoperative radiochemotherapy for the first time, and a significant 5-year survival benefit was observed (23% *vs* 4%)[60]. Based on this small trial, the larger South West Oncology Group/Intergroup trial was established to further evaluate the utility of postoperative radiochemotherapy in the treatment of gastric cancer[61]. In this trial, a total of 556 patients with resectable adenocarcinoma of the stomach or gastroesophageal junction were randomly assigned to surgery alone or surgery plus postoperative radiochemotherapy. The postoperative treatment consisted of fluorouracil/leucovorin, followed by 4500 cGy of radiation (180 cGy for five days). The median overall survival in the surgery-only group was 27 mo, as compared with 36 mo in the radiochemotherapy group; the HR for death was 1.35 (95%CI: 1.09-1.66; $P = 0.005$). The HR for relapse was 1.52 (95%CI: 1.23-1.86; $P < 0.001$). The trial showed promising results for postoperative radiochemotherapy in the treatment of gastric cancer, but the quality control for surgery was still poor, with only a 10% rate of D2 lymphadenectomy. As a result, the value of postoperative radiochemotherapy after D2 lymphadenectomy remained unclear and was not fully accepted in East Asia. Based on the results of this trial, only patients with D0/D1 lymphadenectomy or non-R0 gastrectomy should be assigned to postoperative radiochemotherapy.

Preoperative radiochemotherapy is not currently used as a standard treatment for gastric cancer anywhere. The Chemoradiotherapy for Oesophageal Cancer Followed by Surgery study evaluated the utility of preoperative

radiochemotherapy for esophageal or gastroesophageal junction cancer and reported significant overall survival improvement in the preoperative radiochemotherapy group (49.4 mo *vs* 24 mo; HR = 0.65, 95%CI: 0.495-0.871; $P = 0.003$)[62]. This trial, however, primarily focused on esophageal cancer and does not provide strong evidence for gastric cancer. Other ongoing trials are currently evaluating the safety and utility of preoperative radiochemotherapy (NCT01924819, NCT01815853, NCT00512304, NCT01523015), but a large phase Ⅲ RCT has not yet been reported. The main caveat of this treatment is the potential for radiotherapy to cause tissue damage, leading to undesirable healing of the anastomosis. Before the results of these trials are reported, administration of preoperative radiochemotherapy should be applied with additional attention and care. Based on the results from esophageal cancer studies and small trials, preoperative chemoradiation may bring a favorable survival benefit though a definitive answer requires analysis of the results from the ongoing clinical trials.

PERIOPERATIVE TARGETED THERAPY

Recent advances in molecular therapies have developed a new weapon against AGCs through the use of anti-human epidermal growth factor 2 (HER2) therapies. Trastuzumab, a HER2 monoclonal antibody, was the first drug in the metastatic setting that showed a benefit in overall survival when combined with 5-FU chemotherapy. Assaying the HER2 status of a tumor is imperative to achieve the utmost treatment efficacy. Only HER2 positive (immunohistochemistry [IHC] +++ or fluorescence *in situ* hybridization +/IHC ++) gastric cancer is eligible for trastuzumab treatment. HER2 treatment is a good example for targeted therapy as well as personalized medicine. Although there are not any trials reporting results on the role of trastuzumab in the preoperative setting, a number of case reports with trastuzumab-containing preoperative chemotherapy regimens have been published with promising outcomes, and complete remission has been observed occasionally in these cases[63,64]. The value of perioperative-targeted therapy in clinical practice still needs to be thoroughly evaluated, in addition to the rapid development of molecular oncology.

CONCLUSION

Perioperative treatment is playing an increasingly important role in the multimodal treatment of AGC. Current large-scale RCTs have laid a solid foundation for the utility of perioperative treatment. Several questions still remain. How do we translate these results into clinical practice? How can we present the individual patient with the best benefits and least amount of damage? How do we predict the efficacy of preoperative treatment as early as possible to reduce further damage and decrease costs? Pretreatment evaluation, consisting of a systematic review of tumor stage, location, and biological behavior, is essential to clinical decision-making. Different strategies may be applied on different patient subsets that have been classified by stage, location, biology, and other parameters. Here, we recommend that clinical trial results should be adopted on the appropriate patients based on study inclusion criteria, with regard to age, stage, surgery, and even ethnicity. In addition to the traditional pretreatment workup, new imaging techniques such as PET, diagnostic laparoscopy and DW-MRI, can provide additional information on efficacy prediction and patient selection. Different therapies including preoperative and postoperative chemotherapy and radiochemotherapy are applied in clinical practice and new concepts of perioperative-targeted therapies are starting to play a role in this field. The core of individualized treatment is to use the appropriate strategy on the right patient. Development of molecular biomarkers, molecular and functional imaging techniques will be of great help.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (9): Hepatitis B virus

Chronic hepatitis B in 2014: Great therapeutic progress, large diagnostic deficit

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Abstract

This review analyzes progress and limitations of diagnosis, screening, and therapy of patients with chronic hepatitis B infection. A literature review was carried out by framing the study questions. Vaccination in early childhood has been introduced in most countries and reduces the infection rate. Treatment of chronic hepatitis B can control viral replication in most patients today. It reduces risks for progression and may reverse liver fibrosis. The treatment effect on development of hepatocellular carcinoma is less pronounced when cirrhosis is already present. Despite the success of vaccination and therapy chronic hepatitis B remains a problem since many infected patients do not know of their disease. Although all guidelines recommend screening in high risk groups such as migrants, these suggestions have not been implemented. In addition, the performance of hepatocellular cancer surveillance under real-life conditions is poor. The majority of people with chronic hepatitis B live in resource-constrained settings where effective drugs are not available. Despite the success of vaccination and therapy chronic hepatitis B infection remains a major problem since many patients do not know of their disease. The problems in diagnosis and screening may be overcome by raising awareness, promoting partnerships, and mobilizing resources.

Key words: Chronic hepatitis B; HBsAg; Screening; Liver cirrhosis; Hepatocellular cancer; Entecavir; Tenofovi; Telbivudine; Adefovir; Lamivudine; Interferon

Core tip: This review analyzes progress and limitations of diagnosis, screening, and therapy of patients with chronic hepatitis B. Treatment can control viral replication in most patients today. It reduces risks for progression and may reverse fibrosis. However, screening recommendations have not been implemented, and the performance of hepatocellular carcinoma surveillance is poor. Many patients with chronic hepatitis B live in resource-constrained settings where effective drugs are not available. Despite the therapeutic progress, chronic hepatitis B remains a problem since many patients do not know of their disease. These problems may be overcome by raising awareness, promoting partnerships, and mobilizing resources.

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INTRODUCTION

Infection with the hepatitis B virus (HBV) is a major health problem in many countries around the world. It can lead to chronic hepatitis, liver cirrhosis, hepatocellular cancer (HCC), liver transplantation (LTX), and death[1,2]. Great progress has been made in particular in the fields

of vaccination and therapy. In many industrialized countries three interferons and six nucleot(s)ides (NUC) are now approved for treatment of chronic hepatitis B[3-5]. Oral administration of recent NUC leads to effective, long-lasting suppression of HBV replication[3-5]. In most patients this treatment is without major side-effects; however, it usually does not lead to HBsAg seroconversion and therefore these drugs have to be given for many years or even indefinitely[3-5]. Interferons may lead to HBsAg seroconversion slightly more often when compared with NUC, but they are associated with more side-effects and cannot be given to patients with advanced or even decompensated cirrhosis[3-5]. Overall, treatment of chronic hepatitis B can effectively control viral replication in the long run in almost all patients today[3-5]. It thereby reduces the risk for progression and deterioration of liver disease[6,7] and can even reverse liver fibrosis and initial cirrhosis[8,9]. The treatment effect on reducing the incidence of HCC is less pronounced in particular when cirrhosis is already present[10-14].

Patients with chronic hepatitis B are in general at risk for development of cirrhosis and HCC[15,16]. This risk is associated mainly with serum HBV-DNA (viral load), length of infection, and degree of fibrosis and inflammation. Most cases of HCC develop in cirrhotic livers, but some cases are also seen in patients without cirrhosis. Mortality in patients with HCC is high in particular when the HCC already presents with symptoms. However, despite screening programmes in patients with HBV infection, HCC is often detected at an advanced stage. NUC can be given in patients with advanced or even decompensated cirrhosis and in patients after LTX[3-5]. Unfortunately, the most effective NUC are not approved or reimbursed in several countries with a high prevalence of HBV for economical reasons[17,18].

Despite the success of vaccination and antiviral therapy, chronic HBV infection remains a major problem since many chronically infected patients are unaware of their disease[19-26]. Most of these patients have been born in countries with a high HBV prevalence and have been infected perinatally or in early childhood. In many industrialized countries the majority of patients with chronic HBV infection are migrants from such countries[27]. Even in most industrialized countries there is no systematic screening of high risk groups such as migrants, and in those with screening programs still many patients with chronic hepatitis B are not diagnosed and treated for various reasons.

The present review will focus both on the great therapeutic progress and on the large deficits in diagnosis and screening. It will not discuss vaccination and LTX in greater detail. Also, co-infections with hepatitis C virus (HCV), human immunodeficiency virus (HIV), and hepatitis D virus (HDV) are not covered systematically.

GLOBAL IMPORTANCE AND EPIDEMIOLOGY

Globally, more than two billion people are estimated to have been infected with HBV while more than 240-400 million have chronic HBV infection[1,3]. Approximately 600000 to 1 million people die every year from its consequences[1,28-32]. It is estimated that 15%-25% of perinatally infected subjects will die from HBV related liver disease[1,3]. Because of the global importance of chronic HBV and HCV infection, the WHO organizes the "World Hepatitis Day" on July 28 every year to increase awareness and understanding of viral hepatitis[1]. Many patient support groups and scientific organizations participate in this important event.

The World Health Organization (WHO) has identified three types of regions according to the prevalence of chronic HBV infection: high (> 8%), intermediate (2%-8%), and low (< 2%)[33-35]. High endemicity areas include south-east Asia and the Pacific Basin (excluding Japan, Australia, and New Zealand), sub-Saharan Africa, the Amazon basin, parts of the Middle East, the central Asian Republics, and some countries in Eastern Europe. In these areas 70%-90% of people are infected with HBV before age 40, and 8%-20% are HBV carriers[33]. In countries such as China, Senegal, and Thailand, infection rates are very high in infants and in early childhood with HBsAg prevalence exceeding 25%. In Panama, New Guinea, Solomon Islands, Greenland, and in populations such as Alaskan Indians, infection rates in infants are relatively low and increase rapidly during early childhood[1]. China is estimated to have 120 million people with chronic HBV infection, followed by India and Indonesia with 40 million and 12 million, respectively. North America, Western and Northern Europe, Australia, and parts of South America are considered low endemicity areas with carrier rates < 2% and with < 20% of the population being infected with HBV[1,33,34]. The rest of the world falls into the intermediate range of HBV prevalence with 2%-8% being HBsAg positive[1].

A recent systematic WHO review[36] showed that the prevalence of chronic HBV infection decreased from 1990 to 2005 in most regions of the world, in particular in Central sub-Saharan Africa, Tropical and Central Latin America, South East Asia and Central Europe. The decline of prevalence may be related to the success of HBV immunization. Despite this decrease in prevalence, the absolute number of HBsAg positive persons increased worldwide from 1990 to 2005[36].

DIAGNOSIS AND DEFINITIONS

Acute HBV infection is a clinical diagnosis characterized by symptoms, high serum aminotransferases, and the presence of HBsAg. Usually IgM antibodies against HBc can be detected and HBV-DNA is present. HBeAg can also be detected in most acute infections, but is of little clinical value in this situation. Chronic infection is defined by the persistence of HBsAg for more that 6 mo[3-5]. Patients with chronic HBV infection are usually not diagnosed by clinical disease but by laboratory means. HBsAg is the major tool for screening and diagnosis of

Table 1 Subjects with high risk for HBV infection who should be screened irrespective of symptoms and alanine aminotransferase values[1-5]

All individuals born in areas with high and intermediate HBV prevalence (> 2 % HBsAg positivity) including immigrants and adopted children
Further subjects who belong to a high risk group and should generally be screened
Individuals with elevated ALT/AST or others signs of liver disease
Household and sexual contacts of HBsAg-positive persons
Persons who have ever injected drugs
Persons with multiple sexual partners or history of sexually transmitted disease
Men who have sex with men
Inmates of correctional facilities and jails
Individuals infected with HCV or HIV
Patients undergoing renal dialysis
Patients undergoing chemotherapy or immunosuppressive therapy
All pregnant women

HBV: Hepatitis B virus; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; ALT: Alanine aminotransferase.

chronic HBV infection. HBV-DNA is usually not measured for screening purposes, but determines the risk for major liver disease and the indication for therapy in HBsAg positive subjects. Screening may also include anti-HBc in order to detect rare cases of HBsAg escape variants. Most recent practice guidelines[3-5] recommend HBV screening in the most important risk groups (Table 1).

MODES OF INFECTION AND NATURAL HISTORY

Acute infection with HBV in early childhood is usually asymptomatic and often results in chronic infection. Infection after childhood is usually self-limited and may lead to acute illness with or without jaundice[37-40]. Incubation time varies from 30-180 d with a mean of 75 d. In highly endemic regions perinatal infection is predominant whereas in regions with low endemicity sexual transmission and IV drug abuse are common modes of infection[38-41]. In most countries blood products are today not a significant risk for HBV infection.

The natural history of chronic HBV infection ranges from an inactive carrier with a good prognosis to progressive chronic hepatitis B with high risk for cirrhosis, LTX, HCC, and liver-associated death[1-5,15,16]. Liver cirrhosis develops in 20%-30% of patients with chronic HBV infection[15,16]. Cirrhosis is associated with an HCC risk of approximately 25%, and HBV infection causes 10%-15% of all HCC cases[42]. HBV infection is therefore also associated with an increase in total mortality[43-45] which is due to HCC in about 50% of deaths. Vaccination may reduce disease burden and mortality in industrialized countries[41,46,47]; however, the increased flow of migrants counteracts this trend[1,2]. The prevalence of the HBeAg-negative form of the disease has increased in many areas including Europe over the last 10 - 20 years due to migration processes and predominance of specific HBV genotypes[1,29-32,48-52].

The complication rate of chronic hepatitis B is associated with the degree of viral replication, inflammation, and fibrosis. The risk for cirrhosis is also increased in the presence of fibrosis, a long disease duration, male gender, co-morbidities like alcohol consumption, diabetes mellitus type 2, obesity, and co-infection in particular with HDV or HIV[53-63]. The community-based REVEAL study showed that presence of HBeAg and the detection of HBV-DNA values > 2000 IU/mL are important risk factors for cirrhosis and HCC in an adult Asian population[60-63]. The incidence of HCC is further increased in the presence of cirrhosis and in patients with elevated alanine aminotransferase (ALT) or ALT flares[56-58]. The 5-years cumulative incidence of cirrhosis ranges from 8%-20% after diagnosis, and the 5-year incidence of decompensation is 20% for untreated patients with compensated cirrhosis[29,32,49-52,64-68]. Decompensated cirrhosis is associated with a 14%-35% 5-years survival rate when patients remain untreated[49-50,65]. The yearly incidence of HCC ranges from 2%-6% when cirrhosis is present[63,58,59].

HBV infection may also cause extrahepatic complications such as membranous glomerulonephritis, membranoproliferative glomerulonephritis, IgA-mediated nephropathy, polyarthritis, polyarteritis nodosa, bullous pemphigoid, lichen ruber planus, and cryoglobulinaemia which may be associated with neuropathy (further literature in[69]).

VACCINATION AND FURTHER PREVENTION

A vaccine against hepatitis B has been available since 1982. HBV vaccination has been introduced already in 1982; it is safe and effective. In many countries it is now applied in early childhood in the general population[70]. For regions with high HBV prevalence the WHO recommends that all infants receive active and passive HBV vaccination as soon as possible after birth, preferably within 24 h in order to minimize the risk for perinatal infection. The vaccination after birth should be followed by 2 or 3 doses to complete basic vaccination which induces immunological protection in more than 95% of subjects independent of their age for many years and often lifelong. Hepatitis B vaccine is safe and only rarely has side-effects[71-73]. In countries with a low HBV endemicity WHO and local authorities recommend to vaccinate children and adolescents if they have not been previously vaccinated, as well as all adults with an increased risk of

Table 2 Vaccination recommendations for risk groups[1,35,70]

i.v., drug users
Potential household and sexual contacts with HBV infected people
Subjects frequently requiring blood products, dialysis patients
Patients with organ transplantation
People who are in prisons and correctional facilities for a longer time
People with multiple sexual partners and men who have sex with men
Health-care workers with frequent blood contact
Travellers who are go to highly endemic regions often or for longer time intervals
Clients and staff at institutions for the developmentally disabled
Persons with a history of sexually transmitted infection (STI)
Person without immunoprotection who undergo chemotherapy or immunosuppressive therapy

HBV: Hepatitis B virus.

HBV infection (Table 2).

The WHO states that over one billion doses of hepatitis B vaccine have been used worldwide since 1982. This is a major increase compared with 31 countries in 1992 when the WHO first recommended global HBV vaccination in children. In 2011 a total of 179 WHO Member States regularly vaccinate against hepatitis B, and 93 Member States vaccinated already at birth[1,70]. This progress has decreased perinatal and childhood HBV infection in high endemicity countries from up to 15% to less than 1%. The vaccine is also effective in reducing both the incidence of HCC and mortality from HCC[74-80].

In many countries HBV screening is recommended for all pregnant women. If tested positive for HBsAg, the newborn should receive active and also passive vaccination as soon as possible after birth. In particular the active vaccination markedly reduces the risk for infection from an HBsAg positive mother to her child. The vaccination after birth should be followed by 2 or 3 doses to complete basic vaccination. In pregnant women with a very high HBV replication, it is recommended to consider NUC therapy in the last trimester because in the presence of a high HBV-DNA perinatal infection may occur despite regular vaccination procedures.

For three decades there have been increasingly rigorous blood safety strategies that drastically reduced corresponding HBV infections. In drug users, education and the practice of safe injections significantly reduce the rate of HBV infection. In addition safer sex practices protect against HBV infection; the latter advice is important in particular for sex workers, subjects with multiple sexual partners, and men having sex with men.

SCREENING FOR HBV INFECTION

Although the recent European Association for the Study of the Liver (EASL) and American Association for the Study of Liver Diseases (AASLD) guidelines[3,4] mention important unresolved issues and unmet needs in subjects with chronic hepatitis B infection, they do not focus on the largest deficit; *i.e.*, the high rate of HBV infected patients who do not know of their infection and the corresponding failure of implementation of diagnostic and screening recommendations. As stated by EASL and other guidelines[3-5] little is known as yet about the natural history and indication for treatment in immunotolerant patients with normal or almost normal ALT. There is also a need to better identify those patients in whom NUC therapy can be discontinued. Lastly, there is still a need to develop drugs to enhance the as yet unsatisfactory rate in HBsAg seroconversion.

HBsAg prevalence markedly differs not only between continents, but also in smaller regions like Europe; in the general population it ranges between 0.1% and 5.6% among European countries with high levels seen in particular in Greece and Romania[1,25,81-90]. The Centre for Disease Control (CDC) recommends HBsAg screening for subjects born in countries with a HBsAg prevalence > 2%[91,92]. Similar recommendations have been published for Australia and New Zealand by the local Digestive Health Foundation[93]. Indeed in the USA HBsAg prevalence is 5% higher than in many important migrant populations[22]; prevalence of HBsAg was even 17.4% higher in subjects born in Vietnam[22].

Recent studies estimate that a total of 1.3 million foreign-born persons live in the United States with a chronic HBV infection whereas HBV infection is seen in only 300000 persons born in the United States[94]. Despite the fact that there are up to 2 million patients with chronic HBV infections in the United States, less than 50000 people receive prescriptions for HBV antivirals per year (2.5%). This is probably largely due to the fact that many HBV infected persons remain undiagnosed and/or have no access to the health system[17]. Chronic HBV infection is rarely symptomatic. HBV has been called 'the silent killer' because infected adults often remain undiagnosed and thus untreated until it is "too late"[2]. Among several European countries only approximately 20%-40% of patients knew of hepatitis B at the time of diagnosis[19-26]. In addition, only 27% knew they belonged to a high risk group[19].

The majority of the 240-350 million people with chronic HBV infection, however, live in resource-constrained settings. Here, the problems of missed diagnoses are certainly even higher when compared to industrialized countries. In addition, effective antiviral drugs are not widely available or utilized for HBV infected persons in these regions. Most antiviral agents used for treatment of HIV infection do not adequately suppress HBV, which is of concern for the 10% of HBV/HIV-coinfected subjects in Africa[18]. HBV/HIV-coinfection is often associated with progressive liver disease[18].

To date routine screening recommendations from the NIH (National Institute of Health), the AALSD, and the CDC for all high-risk groups (Table 2) have been expanded to include individuals born in regions with intermediate (28%) and high prevalence (> 8%). Indeed, 45% of people worldwide live in regions with high prevalence and a further 43% in regions of intermediate endemicity[92]. Although health authorities and scientific organiza-

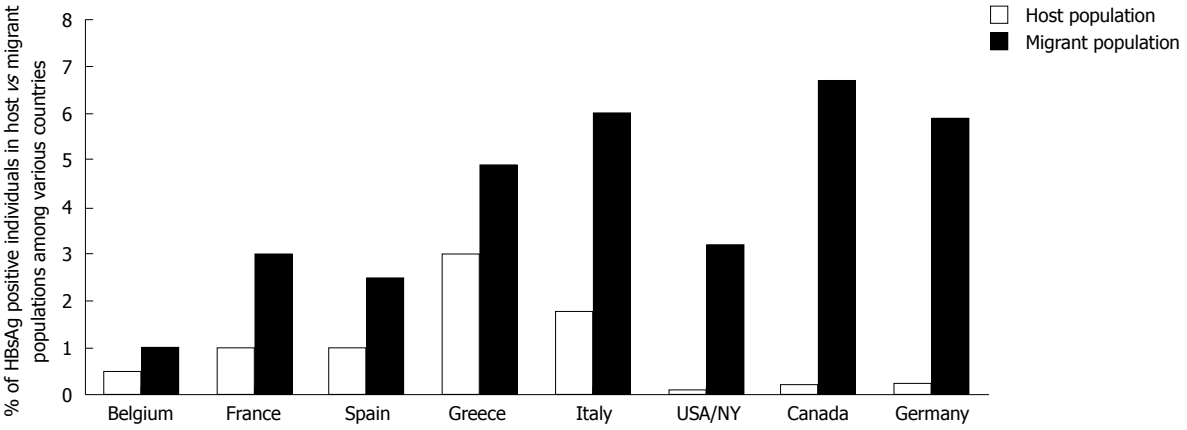

Figure 1 Percentage of HBsAg positive individuals in host versus migrant populations among various countries[27,131-136].

tions recommend to better identification of chronically infected patients born in foreign countries[95,96], as yet such recommendations have not been implemented in systematic or mandatory screening programs.

Chronically infected immigrants are also a reservoir for new infections in host countries[92]. Hepatitis B testing is reliable and inexpensive[92]. HBsAg screening among migrant populations has been shown to be cost-effective[97-100]. Studies have also shown that HBV screening and vaccinating is also effective in infants, children and selected high-risk groups[101].

Similar data have been described for other countries with large migrant populations such as Germany where more than two-thirds of patients with chronic HBV infection have been born in foreign countries with an intermediate or high prevalence of HBsAg[102-107]. Migrants from Turkey account for 22%-33% of all HBV infected people living in Germany. The HBsAg prevalence in the general Turkish population is about 4%; its prevalence may be as high as 7% in Turkish migrants living in Germany[102,103]. Similar data come from the Netherlands, Italy and other European countries[23,107-109]. It has also been shown that HBsAg prevalence is particularly high in large cities and emergency units[110,111]. In all these countries there is no national strategy to implement the screening guidelines[105,106] into daily practice, however.

It is in addition necessary to implement recent guidelines which recommend to screen for HBV (and HCV) in subjects with elevated ALT[3-5]. It has been shown that even this recommendation is not followed by the majority of general practitioners in industrialized countries[112,113], although a large part of undiagnosed HBV infections could be detected by this approach[114]. A recent literature analysis[115] has identified several deficits in migrant screening for viral hepatitis in the European Union (EU). This review and another recent study showed that key factors for a successful screening are support from the local ethnic communities[115,116]. There are obviously also barriers for some migrant populations with already diagnosed HBV infection to have access to treatment and monitoring in industrialized countries[117].

Recent studies showed that knowledge gaps of physicians in hepatitis B diagnosis and management translate into missed opportunities to screen for HBV infection[118-121]. Several studies have shown that antenatal HBsAg screening is cost-effective even in regions with low HBsAg prevalence[122-126]. One study even suggests that general population screening for HBsAg is cost-effective in populations with a prevalence above 0.3%[127]. This study, however, has methodological problems and cannot fully prove its conclusion. Recently it was criticized that immigrants in the United Kingdom are now screened for tuberculosis, but not for HBV infection[128]. In 2007, 194000 of the total of 326000 people with chronic HBV infection in the United Kingdom were born in other countries[129]. In 15 United Kingdom liver centres, 81% of all HBV-infected people were born outside the United Kingdom[130].

Prevalence of HBsAg in migrants from regions with intermediate or high HBsAg prevalence is 5-90 times higher than that in the host population[27,131-136] (Figure 1) and may range up to 15.4% in Albanian refugees in Greece[137-154]. In asylum seekers and refugees in the United Kingdom the HBsAg prevalence ranged from 5.7% to 9.3%[155]. HBsAg prevalence may be even higher than 10% in some migrant ethnic minorities[146-150]. All studies agree that a large proportion of immigrants would benefit from screening for chronic HBV infection given that recent studies have shown that it is cost-effective to screen for chronic HBV infection at a seroprevalence as low as 1%[97-100]. Furthermore, over half of all migrants were found to be susceptible to HBV and could benefit from HBV immunization programs. It was also shown that HBsAg prevalence is even higher in refugees compared to immigrants probably due to experienced violent acts[137,138]. The largest estimated number of HBV-infected migrants live in the United States (1.6 million), Canada (285000), Germany (284000), Italy (201000), the United Kingdom (193000), and Australia (176000)[27].

Immigrants from high endemicity regions are not routinely screened prior to or after arrival in most low endemicity immigrant-receiving countries[139]. Probably due to a high rate of undetected and untreated HBV infection. HCC incidence and mortality are higher in mi-

grants when compared to subjects born in the host country[140,141]. The proportion of immigrants being screened, however, remains low despite these recent recommendations[99,142].

A recent study identified several problems in prevention and control of HBV infection, such as knowledge and awareness gaps in parts of health care and social service providers, high-risk populations, and in the public and policymakers. Ignorance regarding the seriousness of this health problem results in inadequate public resources allocated to HBV prevention and screening programmes[22,143].

Antenatal screening for HBsAg has been introduced in many countries[156-160]. The proportions of pregnant women screened have usually been higher of 90%[135,139,161-166]. All published analyses showed that screening of all pregnant women for HBsAg in order to prevent perinatal infection is highly cost-effective[122-124]. In most countries and regions including the EU there are directives on blood safety including one for screening for HBV[167-169]. This EU report states that in all 33 reporting member states, each donation is tested for HBV and HCV. Some countries have put some effort into increasing the testing for HBV among drug users[170,171] and in men who have sex with men[171]. A study from the Netherlands showed that only 4% of the eligible migrant population with chronic hepatitis B receives treatment also because there is no active screening done[98]. It was further demonstrated that screening for HBV infection in migrants is highly cost-effective[98].

In 2012, the CDC updated its recommendation[172] that HBV infection alone should not disqualify infected persons from the practice or study of surgery, dentistry, medicine, or allied health fields. For those healthcare professionals requiring oversight, the CDC has published specific suggestions for the composition of expert review panels and the threshold value of serum HBV-DNA considered "safe" for practice (< 1000 IU/mL). For most chronically HBV-infected providers and students who conform to current standards for infection control, HBV infection status alone does not require any curtailing of their practices or supervised learning experiences[172].

THERAPY

Acute hepatitis B

There is no specific treatment for acute hepatitis B. There are some hints that patients with severe acute hepatitis B and liver failure may profit from administration of NUC (for further literature please see[173]. Although this concept has not definitely been proven, such treatment is often carried out in many liver centres for patients with acute hepatitis B and liver failure. LTX also still needs to be performed in some patients with fulminant acute hepatitis B.

Treatment goals in chronic hepatitis B

The best treatment goal as yet is HBsAg seroconversion which is considered the closest outcome to clinical cure[3-5,26,174,175]. However, HBsAg seroconversion is infrequently achieved with the current antiviral drugs[3-5,175,176]. In HBeAg-positive patients seroconversion of HBeAg is also considered a desired goal because it is often associated with a low replicative state and an improvement of prognosis[3-5,177]. The long-term, effective suppression of HBV replication is today the most realistic goal and in general is associated with normalization of ALT, histological improvement of inflammation and fibrosis, and a reduction of complications[3-5].

Indications for treatment

Indications for treatment slightly differ between recent EASL *vs* Asian-Pacific Association for the Study of the Liver (APASL) and AASLD guidelines[3-5] (Table 3). In the EASL guidelines, indications for treatment are generally the same for both HBeAg-positive and HBeAg-negative patients; in patients without cirrhosis therapy is considered if HBV-DNA is > 2000 IU/mL for both HBeAg positive and negative patients. In contrast to the EASL consensus, the APASL and AASLD guidelines still differentiate between HBeAg positive and negative patients.

In the APASL and AASLD guidelines[4,5] HBeAg positive patients should be considered for treatment if ALT is greater than two times the upper limit of normal (ULN) or if there is moderate/severe hepatitis on liver biopsy, and if HBV DNA is above 20000 IU/mL (Figure 2). In contrast, in HBeAg-negative patients therapy should be considered if the serum HBV-DNA is above 2000 IU/mL. The APASL and AASLD guidelines recommend consideration for liver biopsy in HBeAg negative patients with HBV-DNA between 2000 and 20000 IU/mL and in those with borderline normal or minimally elevated ALT. In the latter patients, treatment may be initiated if there is moderate or severe inflammation or significant fibrosis on biopsy.

In all guidelines treatment indication is based on the serum HBV-DNA and ALT and on the severity of liver disease which is usually assessed by liver biopsy. Treatment should be considered if HBV-DNA exceeds 2000 IU/mL, ALT levels are above the ULN, and moderate to severe inflammation and/or at least moderate fibrosis is documented by histology. It is mentioned that non-invasive markers may be used instead of liver biopsy once they have been validated in HBV infected patients. Treatment may be initiated also in patients with normal ALT if HBV-DNA is above 2000 IU/mL and if histology shows at least moderate inflammation and fibrosis.

In the EASL guidelines[3], need for liver biopsy and treatment should be considered separately in immunotolerant patients. These patients, defined as HBeAg positive subjects under 30-40 years of age with persistently normal ALT levels and high HBV-DNA levels without evidence of liver disease and without a family history of HCC or cirrhosis, do not require immediate liver biopsy or therapy. These patients should, however, be monitored every 3-6 mo[178,179]. Liver biopsy and therapy should be

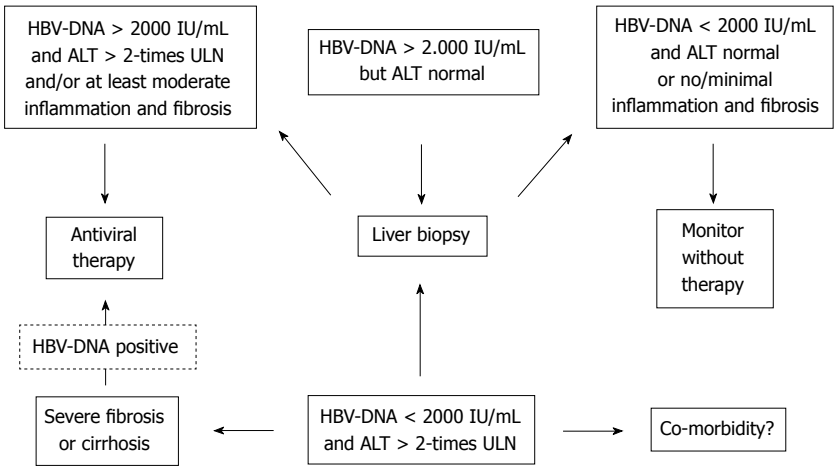

Figure 2 Treatment algorithm modified from recent guidelines[3-5].

Table 3 Indication for treatment in European Association for the Study of the Liver, Asian Pacific Association for the Study of the Liver, and American Association for the Study of Liver Diseases guidelines[3-5]

EASL
Indication similar for both HBeAg-positive and HBeAg-negative patients
Consider therapy if HBV-DNA is > 2000 IU/mL, ALT is > ULN and there is moderate to severe active necroinflammation and/or at least moderate fibrosis on liver biopsy
Consider biopsy and therapy separately in immunotolerant patients: HBeAg-positive patients < 30 yr with persistently normal ALT and high HBV-DNA, without evidence of liver disease and family history of HCC or cirrhosis, do not require liver biopsy or therapy. Follow-up is mandatory
Consider biopsy or therapy in patients > 30 yr and/or with a family history of HCC or cirrhosis
HBeAg-negative patients with persistently normal ALT and HBV-DNA of 2000-20000 IU/mL and without evidence of liver disease do not require liver biopsy or therapy. Follow-up is mandatory
Patients with ALT > 2 times ULN and HBV-DNA >20000 IU/ml may start treatment without biopsy
Therapy indicated in compensated cirrhosis and detectable HBV-DNA even if ALT is normal
Patients with decompensated cirrhosis and any detectable HBV-DNA require urgent therapy
APASL
HBeAg positive: consider therapy if ALT >2 times ULN and HBV DNA > 20000 IU/mL
HBeAg-negative: consider therapy if ALT > 2 times ULN and HBV DNA > 2000 IU/mL
Consider therapy in advanced fibrosis or cirrhosis with any ALT level
Therapy in all patients with decompensated cirrhosis independent of HBV-DNA
Therapy in compensated cirrhosis if HBV-DNA is > 2000 IU/mL
In the absence of cirrhosis/severe fibrosis patients with persistently normal or minimally elevated ALT should not be treated irrespective of the height of HBV-DNA. Follow-up is mandatory
AASLD
HBeAg-positive: consider therapy if ALT >2 times ULN with moderate/severe hepatitis on biopsy and HBV-DNA > 20000 IU/mL
In general no therapy if ALT is persistently normal or minimally elevated (< 2 times ULN); consider biopsy in patients with fluctuating /minimally elevated ALT especially in those > 40 yr; consider therapy if there is moderate or severe necroinflammation or significant fibrosis on biopsy
HBeAg-negative: consider therapy if HBV-DNA > 20000 IU/mL and ALT > 2 times ULN
Consider biopsy if HBV-DNA is 2000-20000 IU/mL and ALT is borderline normal or minimally elevated. Consider therapy if there is moderate/severe inflammation or significant fibrosis on biopsy

HBV: Hepatitis B virus; EASL: European Association for the Study of the Liver; APASL: Asian-Pacific Association for the Study of the Liver; AASLD: American Association for the Study of Liver Diseases; ALT: Alanine aminotransferase.

considered in those immunotolerant patients older than 30-40 years and/or with a family history of HCC or cirrhosis[4-5,180,181]. More frequent monitoring and/or liver biopsy should be performed when ALT levels become elevated[4,182-185].

Thus, differences in the guidelines particularly refer to patients with a HBV-DNA between 2000 and 20000 IU/mL and minimally elevated or almost normal ALT (Figure 2). All guidelines[3-5] actually recommend that such patients should be monitored closely and also should be considered for liver biopsy in order to further clarify the need for treatment. Patients with persistently normal ALT often have minor histological changes and due to the APASL guidelines may not need treatment[5] urgently unless they have advanced fibrosis or cirrhosis[186,187]. All guidelines[3-5] agree that these patients should be monitored every 3-6 mo in order to not to oversee ALT flares. Patients with HBV-DNA > 20000 IU/mL and normal ALT should be monitored even more closely than those with lower HBV-DNA.

HBeAg negative patients with persistently normal ALT and HBV-DNA levels between 2000 and 20000 IU/mL and without any evidence of liver disease also do not require immediate liver biopsy or therapy. Monitoring

every 6-12 mo is considered mandatory in this group.

HBeAg-positive and HBeAg-negative patients with ALT above two times ULN and serum HBV-DNA above 20000 IU/mL may start treatment even without a liver biopsy. In such patients, liver biopsy may provide additional useful information, but it does not usually change the decision for treatment[3-5]. A non-invasive method for the estimation of the extent of fibrosis and most importantly to confirm or rule out cirrhosis is considered useful in patients who start treatment without liver biopsy. If liver biopsy is not feasible, noninvasive assessment of liver fibrosis is an alternative[4,56,57,188,189]. Age, health status, family history of HCC or cirrhosis, and extrahepatic manifestations also need to be considered when treatment indication is evaluated.

Indications for treatment also slightly differ between recent EASL *vs* APASL and AASLD guidelines for patients with compensated cirrhosis. In the EASL guidelines these patients must be considered for treatment if HBV-DNA is detectable even though ALT levels are normal. In contrast APASL and AASLD guidelines[4,5] recommend treatment in patients with compensated cirrhosis only if HBV-DNA is above 2000 IU/mL. This difference in guidelines is due to the fact that it is unknown whether cirrhotic patients with a HBV-DNA between 20 and 2000 IU/mL will benefit from antiviral therapy. There is no doubt that cirrhotic patients have the highest risk for decompensation and HCC; thus the author strongly supports the EASL recommendation[3] to treat all cirrhotic patients with detectable HBV-DNA independent of the level of HBV-DNA and ALT.

All guidelines[3-5] recommend that patients with decompensated cirrhosis and detectable HBV-DNA require urgent antiviral treatment with a NUC with a high antiviral efficacy and a high resistance barrier. Such effective antiviral therapy in some patients may lead to improvement in those with decompensated cirrhosis, while other patients still need evaluation for LTX[190,191].

TREATMENT STRATEGIES

In general, there are two different strategies to treat chronic hepatitis B[3-5]: (1) pegylated Interferon (PEG-IFN) may be used with a finite duration; or (2) NUC are usually given without a finite duration as a long-term treatment.

Although EASL guidelines[3] mention that NUC may also be used for a finite duration, in many patients these substances have to be given for an indefinite duration. EASL guidelines recommend that NUC aimed for a finite treatment should have the highest barrier to resistance to rapidly reduce levels of viremia to undetectable levels and avoid breakthroughs due to HBV resistance[3]. In HBeAg positive patients NUC (as well as interferons) may lead to HBeAg seroconversion which may be associated with a decrease of HBV-DNA to a low replication state. In such patients there may be no need for further antiviral treatment if they remain in a low replicative state after HBeAg seroconversion. In most series, however, less than 30% of patients will have such seroconversion during and after treatment with NUC or interferons[3-5,192-198]; in addition, some seroconverted patients will later either reseroconvert to a positive HBeAg or may have a level of HBV-DNA and ALT requiring further treatment. When HBeAg seroconversion occurs during NUC treatment, therapy should be prolonged for an additional 12 mo[194]; a durable off-treatment response (persistence of anti-HBe seroconversion) may then range from 40%-80%[192-198]. In any case, all patients require close virological monitoring after treatment cessation following HBeAg seroconversion.

There are some hints that HBeAg seroconversions after interferon therapy are more frequent and may be more durable when compared with NUC due to a better immune-mediated control of HBV infection[199-204]. Rates of HBeAg seroconversion due to therapy with PEG-IFN approach 30% and those due to NUC 20%[190,191,205-221]. In patients adherent to treatment, virological remission rates of > 90% can be maintained with ongoing entecavir or tenofovir for up to 8 years[222-224].

PEG-INTERFERON

Only PEG-IFN α2a is approved for treatment of chronic hepatitis B; it has largely replaced standard interferon (IFN) mainly due to practical and convenience reasons (injection only once weekly). Recent guidelines recommend that PEG-IFN should be given in general for 48 wk, preferably in HBeAg positive patients with good chances for HBeAg seroconversion. It is probably less effective in HBeAg-negative patients. Because of a higher risk of side-effects and inconvenience associated with (PEG)-IFN *vs* NUC, the decision about the antiviral agent should be discussed with the individual patient in detail. Combinations of interferon or PEG-IFN with NUC has not shown long-term advantage over corresponding mono-therapies[225,226] and are neither approved by the drug agencies nor recommended by guidelines[3-5].

Since interferon often has side-effects, one would like to stop treatment if there is only little probability for response. Such predictors of response to IFN-based therapy include baseline and on-treatment factors. High baseline ALT and low baseline HBV-DNA are associated with a higher response rates; HBV genotypes and IL28B genotypes are also associated with HBeAg and HBsAg seroconversion[191,199,202,224]. On-treatment HBsAg levels and the kinetics of its decline are good predictors of sustained response to Peg-IFN.

HBeAg seroconversions at 6-mo post-treatment are higher in patients with HBsAg levels < 1500 IU/mL at weeks 12 and 24 when compared with those with HBsAg levels > 20000 IU/mL at the same time points (57% *vs* 16% at week 12, and 54% *vs* 15% at week 24)[215,222]. Patients without a decline of HBsAg at week 12 had a 82%-97% probability of non-response during post-treatment follow-up[227-231]. Therefore, stopping PEG-IFN

therapy may be considered in the latter situation[3,4].

Peg-IFN treatment is probably less useful in HBeAg negative patients since treatment goals are ill defined; only some patients remain in a low replicative state after treatment with PEG-IFN for 48 wk. HBeAg negative patients who are treated with PEG-IFN and fail to achieve any decline in HBsAg levels or have a HBsAg > 20000 IU/mL at week 12 have a very low probability of response; therefore, PEG-IFN therapy may be stopped at that time[232,233].

(PEG)-Interferon(s) are associated with more side-effects than NUC and they are contraindicated in decompensated HBV-associated cirrhosis, autoimmune diseases, severe depression/psychosis, and in pregnant women[3-5].

ORAL THERAPY IN HBV INFECTION

Today entecavir and tenofovir are the most potent HBV inhibitors with a high barrier to resistance[210,213,221,223,224,234] in treatment-naïve patients. Thus, they can be confidently used as first-line monotherapies[3-5]. Patients with resistance or failure to lamivudine or telbivudine should receive tenofovir if treatment is indicated[3,4].

According to EASL guidelines[3] the further three NUC lamivudine, telbivudine and adefovir may only be used if more potent drugs with high barrier to resistance are not available. Similar to the EASL guidelines[3], AASLD guidelines[5] also state that PEG-IFN, tenofovir or entecavir are preferred for initial treatment. APASL guidelines[4] recommend that the decision as to which agent to be used should be an individual one, based on disease severity, history of flares, hepatic function, the rapidity of drug action, resistance profile, side effects, drug costs, and patient choice. Cost-effectiveness of drug therapy is specific for each country and should be studied independently to guide the choice of drug. The EASL also states that entecavir or tenofovir is the preferred NUC[3].

According to guidelines[3-5], IFN-based therapy is preferred in younger patients. IFN-based therapy has more side effects and requires closer monitoring. For patients with ALT level > 5 times ULN, NUC are recommended if there is a concern about hepatic decompensation[4]. IFN-based therapy is also effective in patients with a higher ALT level if there is no concern about hepatic decompensation. For HBeAg-positive patients with an ALT level between 2 and 5 times ULN, the choice between IFN-based therapy and NUC is less clear, and either agent may be used[4].

Lamivudine was the first NUC approved for treatment of hepatitis B and it is now the most inexpensive agent. However, it is associated with high rates of resistance[235-238]. Thus, the European Medicines Agency (EMA) advises to not use lamivudine any longer for initiation of therapy.

Adefovir was the second NUC approved for treatment of hepatitis B; it is also associated with risks of resistance. Thus, for some time the combination of lamivudine and adefovir was used for patients with resistance problems[213,224,239].

Similar to lamivudine, telbivudine has a relatively low barrier to resistance. Resistance rates are high in particular in patients with high baseline HBV-DNA levels and in those with detectable HBV-DNA after 6 mo of therapy[211,220]; resistance rates to telbivudine are relatively low in patients with a baseline HBV-DNA < 2×10^8 IU/mL for HBeAg-positive and < 2×10^6 IU/mL for HBeAg-negative patients who achieve a negative HBV-DNA 6 mo after beginning treatment[220,240].

In most patients NUC need to be given as a long-term treatment. Only 20%-30% of HBeAg positive patients have an HBeAg seroconversion and some of them may not need long-treatment. HBeAg negative patients require long-term treatment because most of them have a relapse of HBV replication after stopping of therapy. The rate of HBsAg seroconversion in HBeAg negative patients under treatment with NUC is very low. Long-term treatment is also recommended for patients with cirrhosis irrespective of HBeAg status or anti-HBe seroconversion on treatment. EASL guidelines[3] recommend that the most potent drugs with the optimal resistance profile, *i.e.,* tenofovir or entecavir, should be used as first-line mono-therapies. Whatever drug is used, HBV-DNA should effectively be suppressed[3-5].

There are as yet no data to indicate an advantage of de novo combination treatment with NUC in NUC-naïve patients receiving either entecavir or tenofovir[241].

In patients with lamivudine resistance a switch to tenofovir is recommended[3]. In patients with adefovir resistance recommendations consider the prior treatment: If the patient was not previously treated with NUC a switch to entecavir or tenofovir is recommended with entecavir preferred in patients with high viraemia: if the patient had prior lamivudine resistance, a switch to tenofovir and addition of a nucleoside analogue is recommended. In the presence of telbivudine or entecavir resistance a switch to or addition of tenofovir is feasible. Tenofovir resistance has not been described as yet.

MONITORING UNDER NUC TREATMENT

The monitoring under NUC treatment is described in detail in current guidelines[3-5]. In general HBV-DNA and other laboratory values need to be checked every 3 mo after after the beginning of therapy. Undetectable HBV-DNA by real-time PCR (*i.e.*, < 10-15 IU/mL) should be achieved to avoid resistance. Once HBV-DNA remains undetectable and ALT normal, the regular interval may be expanded to 6 mo. Treatment with tenofovir or entecavir is preferred in all guidelines because of their potency and minimal risk of resistance[3-5,242,243]. The dose of NUC needs to be adjusted if the estimated creatinine clearance is reduced. Thus, renal function and serum phosphate need to be monitored in particular with adefovir and tenofovir treatment[244].

Patients with cirrhosis require intensively careful

monitoring for resistance and flares in order to stabilize patients and to prevent the progression to decompensated liver disease[245,246]. Regression of fibrosis and even reversal of Child A cirrhosis have been reported in patients with prolonged suppression of viral replication[8,9].

Patients with decompensated cirrhosis should be treated in liver centres with a backup of liver transplantation. Antiviral treatment is indicated irrespective of the HBV-DNA level in order to prevent reactivation[190,191,205]. Entecavir or tenofovir should be used since they are effective and safe in this subgroup[190,191,205]. If patients with decompensated cirrhosis show clinical improvement LTX may be avoided. In such patients indefinite treatment with entecavir or tenofovir is recommended. Patients who need LTX should also be treated continuously with entecavir or tenofovir to ensure maximal suppression of HBV-DNA at the time of LTX and thus to reduce the risk of HBV recurrence[247-249]. Long-term monitoring for HCC is necessary despite virological remission since there is still a risk of developing HCC in particular in patients with pre-existing cirrhosis[7,10,250].

SURVEILLANCE OF HCC

HCC is the sixth most common cancer in the world and the third most common cause of cancer mortality[251]. HCC is the most devastating outcome of chronic HBV infection and it is often diagnosed in late stages with little curative chances. Overall 5-years survival is only approximately 5%; in subgroups of HCC patients with an early diagnosis, 5-year survival may be improved to 40%-70%[252]. However, only a small number of patients are diagnosed at stages early enough to have a chance for cure. In population-based U.S. studies, only approximately 10% of HCC patients receive treatment with such a curative approach[253]. Therefore, HCC surveillance has been advocated to detect HCC at an early stage in order to assure a curative approach[3-5,254]. All three current major international HCC guidelines[255-257] and all three major international HBV guidelines[3-5] recommend to perform surveillance for HCC in high-risk populations. These recommendations also consider the cost-effectiveness of such screening which depends on the HCC incidence in the target populations. According to the AASLD guidelines surveillance is cost-effective if the expected HCC risk exceeds 1.5% per year in patients with hepatitis C and 0.2% per year in patients with hepatitis B[257].

The efficacy and cost-effectiveness of surveillance depend on the incidence of HCC in the target population. The HCC risk increases in HBV-infected patients with the degree of fibrosis, age > 40-50 years, duration of infection, level of HBV-DNA, inflammatory activity, co-infections, co-morbidities, and a family history of HCC. Thus, it is rather difficult to exactly identify all subgroups of HBV-infected patients who should be screened[258]. In addition, thresholds for cost-effectiveness of surveillance programs vary according to the economic situation of each country[257]. In any case, all guidelines agree that all patients with HBV infection and cirrhosis or severe fibrosis should be included in HCC surveillance programmes. One large controlled randomized study from China has proved the benefit of surveillance in more than 18000 patients with chronic HBV infection[258]. Surveillance with ultrasound and alpha fetal protein (AFP) measurement every 6 mo reduced HCC mortality by 37% despite of the fact that the compliance of scheduled tests was only 58.2%[258]. Former guidelines recommended HCC surveillance using ultrasound and serum AFP every 6 mo for high-risk HBV-infected individuals (cirrhosis, males, age > 40 years, positive family history of HCC, high viral replication)[31,254].

Most recent studies have shown that the sensitivity, specificity, and diagnostic accuracy of ultrasound are higher than those of AFP (for detailed literature please see[25-257]. Ultrasound is the most widely used imaging technique used for HCC surveillance with an acceptable sensitivity (58%-89%) and a specificity of up to 90%[259,260]. A meta-analysis including 19 studies showed that ultrasound surveillance detected the majority of HCC tumours before they presented clinically with a sensitivity of 94%[261]. However, ultrasound was less effective for detecting early-stage HCC with a sensitivity of only 63%[262]. In contrast, in a Japanese cohort ultrasound surveillance performed by skilled physicians resulted in a mean size of the detected tumours of < 2 cm with less than 2% of the cases exceeding 3 cm[263]. The widespread use of ultrasound relies on its non-invasiveness, good acceptance by patients, and moderate costs. However, detection of a small HCC in a cirrhotic liver may be difficult even for a skilled investigator[255].

AFP is still the most widely tested tumour marker in HCC. It is known that persistently elevated and increasing AFP values indicate a risk for HCC development[264]. However, AFP is not very useful for surveillance purpose. AFP is often unreliable in patients with chronic hepatitis B because of fluctuating levels which also depend on the inflammatory activity[265]. In addition, a considerable proportion of HCC is not associated with any AFP elevation[266-269]. For diagnostic purposes, an AFP cut-off of 20 ng/mL showed good sensitivity but low specificity, whereas a cut-off of 200 ng/mL was associated with a sensitivity of only 22%[270]. A randomized study[271] and a population-based observational study[265] showed controversial results concerning the use of AFP measurements. The randomized study showed that screening with AFP resulted in earlier diagnosis of HCC, but not in any overall reduction in mortality, because therapy for the patients found by screening was ineffective[271]. The second prospective population-based study determined HCC screening in HBV-infected Alaskan natives with AFP determination done every 6 mo[265]. Subjects with an elevated AFP level were then evaluated for the presence of HCC by ultrasound[265]. In the latter study screening with AFP every 6 mo was effective in detecting most HCC tumours at a resectable stage and significantly prolonged survival rates when compared with historical controls in this population[265].

Most recent studies agree that AFP determination

Table 4 Surveillance of hepatocellular carcinoma in subjects with chronic hepatitis B virus infection[3-5,31,255-266]

Surveillance all patients with cirrhosis and severe fibrosis
Including those treated with NUC
Surveillance in individuals with increased HCC risk, even without cirrhosis or severe fibrosis
High HBV-DNA
Males
Age > 40-50 yr (in particular in Asia and Africa)
Long duration of infection
Significant inflammation
Co-infection with HIV, HCV and HDV
Co-morbidities (*e.g.*, diabetes mellitus, high alcohol consumption, NASH/NAFDL)
For surveillance: Ultrasound done every 6 mo by a skilled physician
Determination of AFP (in combination with ultrasound) still recommended by APASL[257], but not by EASL and AASLD guidelines[255-256]
AFP is less useful than ultrasound for surveillance of HCC

HCC: Hepatocellular cancer; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; HDV: Hepatitis D virus; AFP: Alpha fetal protein.

lacks adequate sensitivity and specificity for surveillance as well as for diagnosis[262-264,266-273]. Thus, according to AASLD and EASL guidelines[255,256] surveillance has to be based mainly on ultrasound examination. Better tumour markers need to be developed for HCC surveillance[255,256]. As yet, AFP-L3 and DCP are also not considered useful for routine practice[255,256].

The recommended surveillance interval is still 6 mo[255,256]. Candidates for surveillance include HBV-infected subjects with cirrhosis or severe fibrosis, Asian men > 40 years and Asian women > 50 years, subjects with a family history of HCC, Africans > 20 years, and all subjects > 40 years with persistent or intermittent ALT elevation and/or HBV DNA level > 2000 IU/mL[256]. Screening with AFP should be considered only when ultrasound is not readily available[256,265]. When combined with ultrasound, AFP led only to HCC detection in an additional 6%-8% of cases not identified by ultrasound alone[265]. The best interval of HCC surveillance for HCC depends on the rate of tumour growth and on the tumour incidence in the target population. According to the estimated doubling time of HCC volume[274-277], a 6-mo interval is most often used in clinical practice and recommended by recent guidelines[3-5,255-257]. Some studies comparing the efficacy of 6-mo *vs* 12-mo intervals showed a similar efficacy[265,278,279] whereas other studies showed a better one with the 6-mo intervals[280,281]. A shorter 3-mo interval has been proposed for patients with a very high HCC risk and by Japanese guidelines[282,283]. In contrast to the latter guideline recommendation, there is no evidence that surveillance with a 3-mo interval is better than with a 6-mo interval[284].

Groups at high risk include all patients with severe fibrosis or cirrhosis in all guidelines [3-6,255-257]. In general, however, all patients with chronic HBV infection are at some risk of HCC development. Unfortunately, the risk of HCC development is less well established in non-cirrhotic individuals[255-257]. In Caucasian patients the HCC risk is mainly restricted to patients with cirrhosis[285,286]. According to EASL and AASLD guidelines[255,256] is it unclear whether these patients should have any HCC surveillance. Thus, surveillance is not generally recommended in Caucasian non-cirrhotic patients when viral replication and inflammation are low[255,256,287-291]. The HCC risk, however, is increased also in non-cirrhotic Caucasians with older age, higher viral replication, co-infection (such as HCV, HDV and HIV) and concomitant other liver diseases[255,256]. Such subgroups of adult non-cirrhotic Caucasian patients should also undergo surveillance in addition to all cirrhotic patients[255,256] (Table 4).

In contrast to Causasians, Asian patients without cirrhosis, however, appear to have a higher risk for HCC regardless of the HBV-DNA values[58,287,292,293] and thus should undergo surveillance[256,294]. The annual HCC risk in Asian patients exceeds 0.2% when HBV-DNA is > 2000 IU/mL[295].

The incidence of HCC in male Asian patients starts to exceed 0.2% at age 40[296]; therefore HBV-infected Asian men above age 40 should undergo surveillance; according to AASLD guidelines Asian women should undergo surveillance above age 50[256]. Interestingly, the APASL guidelines recommend general surveillance only in cirrhotic patients[257]. In the presence of a history of a first-degree relative with HCC, surveillance should start at a younger age[296,297] while the exact age still needs to be defined. Africans with hepatitis B also seem to get HCC at a younger age than Caucasians and thus should undergo surveillance at a younger age[298,299].

Risk scores have been developed to better identify those HBV-infected patients who should have surveillance. The score by Yuen *et al*[300] includes factors such as male gender, older age, higher HBV-DNA levels, core promoter mutations, and cirrhosis. A similar score has been based on the data from the REVEAL study[301,302]. Both scores are not ready to be used in clinical practice[256].

The need for surveillance under long-term antiviral treatment with NUC is not well defined as yet. Several studies suggest that long-term effective NUC treatment will reduce but eliminate the HCC risk which is still present in males, in patients > 40 years of age and in those with cirrhosis[10-14,303-305]. Thus, the latter patients should undergo HCC surveillance even if the HBV-DNA is undetectable and ALT values are normal under long-term NUC treatment[255,256].

In summary, the more recent EASL and AASLD guidelines[255,256] recommend to use ultrasound as the only surveillance tool; AFP is not considered useful for this purpose. Only the recent APASL guideline[257] still recommends to carry out both ultrasound and APF measurement every 6 mo for HCC surveillance in cirrhotic patients with chronic HBV infection.

PERFORMANCE OF HCC SURVEILLANCE UNDER REAL-LIFE CONDITIONS

The performance of HCC surveillance under real-life

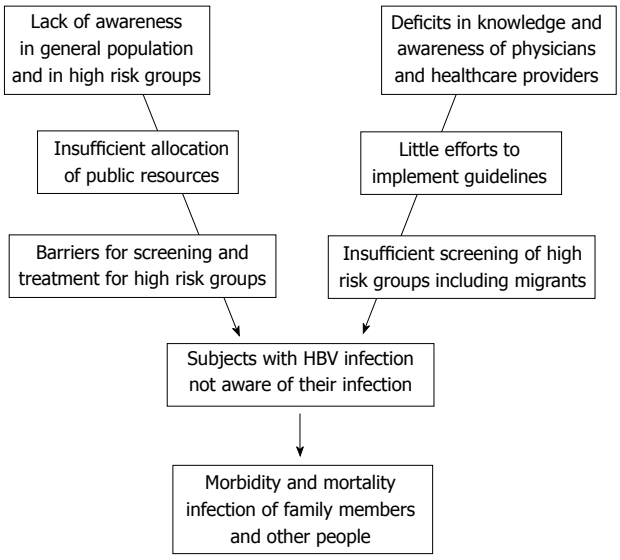

Figure 3 Problems in diagnosis and screening of chronic hepatitis B virus infections.

conditions is poor both for HBV- and HCV-infected individuals. In a recent study of 13002 HCV-infected veterans diagnosed with cirrhosis during 1998-2005, only 12% received annual surveillance in the 3 years following their cirrhosis diagnosis, and less than 50% received a surveillance test in the first year following diagnosis of cirrhosis[306]. At least three retrospective studies among patients with a newly diagnosed HCC reported very low rates of surveillance prior to the HCC diagnosis[307,308]. In one of these studies only 29%[308] of subjects received annual surveillance in the 3 years before the diagnosis of cirrhosis[308]. These deficits likely reflect both knowledge gaps in guidelines as well as logistical problems such as lack of recall systems. Direct patient involvement in HCC surveillance has been shown to improve performance of surveillance[309]. Also, implementation of quality measures incorporating automatic recall systems for providers may improve HCC surveillance[310].

CONCLUSION

Despite the success of vaccination and the progress of antiviral therapy, chronic HBV infection remains a major health problem since many patients do not know of their disease. There is a corresponding lack of awareness both in the general population and in high risk groups (Figure 3). As yet the allocation of public resources is insufficient to increase the awareness and to implement national and international guidelines. In addition deficits in knowledge and awareness of physicians and healthcare providers have been identified. The performance of HCC surveillance under real-life conditions is also poor. All these problems add to barriers for screening and treatment of high risk groups and finally increase the risk for morbidity and mortality of HBV-infected individuals who, in addition, might infect family members and other people. These problems may be overcome by raising awareness, promoting partnerships, and mobilizing resources.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (9): Hepatitis B virus

Control of hepatitis B virus replication by interferons and Toll-like receptor signaling pathways

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Abstract

Hepatitis B virus (HBV) infection is one of the major causes of liver diseases, affecting more than 350 million people worldwide. The interferon (IFN)-mediated innate immune responses could restrict HBV replication at the different steps of viral life cycle. Indeed, IFN-α has been successfully used for treatment of patients with chronic hepatitis B. However, the role of the innate immune response in HBV replication and the mechanism of the anti-HBV effect of IFN-α are not completely explored. In this review, we summarized the currently available knowledge about the IFN-mediated anti-HBV effect in the HBV life cycle and the possible effectors downstream the IFN signaling pathway. The antiviral effect of Toll-like receptors (TLRs) in HBV replication is briefly discussed. The strategies exploited by HBV to evade the IFN- and TLR-mediated antiviral actions are summarized.

Key words: Hepatitis B virus; Interferon; Toll-like receptor; Interferon stimulated genes; Innate immune response

Core tip: Hepatitis B virus (HBV) infection is one of the major causes of liver diseases affecting more than 350 million people worldwide. Interferon (IFN)- and Toll-like receptors (TLR)-mediated innate immune responses could restrict HBV replication at the different steps of viral life cycle. Though a great number of publications in this field appeared during the last years, there is no review to discuss the progress. Here, we summarized the currently available knowledge about the anti-HBV effect of IFNs and TLRs and the possible effectors downstream the IFN signaling pathway. This review provides an overview for scientists working on HBV and related fields.

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INTRODUCTION

The hepatitis B virus (HBV) is a member of the *Hepadnaviridae* family and an enveloped virus with a partially double stranded DNA genome[1]. Primary HBV infection in about 90% of infants infected at birth, 20%-50% of children, and 5% of adults will progress to chronic course of infection[2]. Chronic HBV infection is a global public health problem, affecting more than 350 million people worldwide[3]. The risk of developing severe liver diseases such as cirrhosis and hepatocellular carcinoma is increased in patients with chronic hepatitis B[3]. Interfer-

ons (IFNs) and nucleoside analogues are the commonly used drugs for anti-HBV treatment.

The innate immunity is the first line of active host defense against viral infection. The recognition of viral pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), RIG-I like receptors (RLRs), NOD-like receptors (NLRs), and others triggers signals to activate intracellular pathways and leads to the production of antiviral and immune regulatory effector molecules. However, HBV was believed to be a stealth virus since it did not trigger or only triggered a limited innate response in infected Chimpanzees and patients during the acute phase of infection[4-6]. The HBV-specific T cell response is thought to be essential for the control of HBV infection. Besides the classical way to remove infected hepatocytes by cytolytic mechanisms, HBV may also be cleared *via* noncytopathic mechanisms involving antiviral cytokines such as IFN-γ and TNF-α[7-9]. Though HBV does not trigger type I IFN production in hepatocytes and in the infected liver, IFN-α and -β are shown to be able to suppress HBV replication *in vitro* and in HBV transgenic mouse models. Recombinant IFN-α has been approved and successfully used as a standard treatment for chronic HBV infection. Here, we summarize the available data about IFN-mediated anti-HBV actions and the control of HBV by TLR activation.

HBV LIFE CYCLE

The full HBV infection cycle could only be studied in primary human and Tupaia hepatocyte cultures before the recent discovery of the HBV receptor[10]. The viral entry is supposed to be a receptor mediated endocytosis process or a membrane fusion process[11]. Two elements of the envelop protein are required for HBV infection, the receptor binding site located within the N-terminal pre-S1 domain and the infectivity determinant in the surface-exposed antigenic loop[12-14]. Recently, sodium taurocholate cotransporting polypeptide (NTCP) was identified as a functional receptor mediating HBV entry[15]. After the entry, the endosomal vesicles play an important role in uncoating and release of viral capsids containing HBV genome. The transport of viral particles to the late endosomes is required in the early step of HBV infection[16]. After uncoating, HBV capsid is actively transported to the nucleus in a microtubule-dependent manner[17]. It is proposed that HBV capsid is disassembled in the nuclear basket to release HBV genome into the nucleus[18,19]. In the nucleus, HBV genome, the partially double-stranded viral relaxed circular DNA (rcDNA), is repaired by both viral and cellular enzymes and converted to covalently closed circular DNA (cccDNA). The cccDNA is in complex with cellular proteins like histones and other regulatory proteins and organized as a viral minichromosome[20]. The episomal cccDNA then serves as template for viral transcripts, including the 3.5 kb (pregenomic (pg) RNA), 2.4 kb, 2.1 kb, and 0.7 kb mRNAs. The transcription is under the control of four promoters (the core, pre-S1, pre-S2/S, and X promoters) and two enhancers (En Ⅰ and En Ⅱ). The HBV mRNAs encode different proteins: the 3.5 kb mRNA for HBV polymerase (pol), HBcAg and HBeAg, the 2.4 kb and 2.1 kb mRNAs for the large, middle, and small surface proteins (L, M and S HBsAg), and the 0.7 kb mRNA for HBx protein[21]. HBcAg encapsidates the pgRNA and pol protein and forms the immature nulceocapsid. Within the capsid, the pgRNA is first reverse transcribed to minus strand DNA; meanwhile, the pgRNA is degraded by a ribonuclease H which is a domain of HBV pol protein. The plus strand of HBV DNA is only partly synthesized using the minus strand as the template to form the relaxed circle DNA form of HBV genome. The mature HBV capsids may be either recycled to the nucleus for amplification of the cccDNA pool or be enveloped in HBsAg to form infectious virions and released from hepatocytes[22,23].

CONTROL OF HBV REPLICATION BY IFN-MEDIATED ANTIVIRAL MECHANISMS

IFNs act through binding to the cellular receptors and are able to induce a great numbers of genes termed as IFN-stimulated genes (ISGs)[24]. The ISGs have diverse functions and could inhibit HBV replication at different steps as discussed in detail below. Due to the lack of efficient *in vitro* infection system for HBV, the antiviral effect of IFNs on the entry and uncoating steps of HBV life cycle has not been studied so far. IFN-mediated antiviral functions against HBV were mainly examined in human hepatoma cells transiently or stably transfected with replication-competent HBV genomes and HBV transgenic mouse models. In addition, IFNs have immunomodulatory functions which will not be discussed in the present review[25,26] (Figure 1 and Table 1).

IFN-α induces epigenetic modification of cccDNA minichromosomes and inhibits cccDNA transcription

HBV cccDNA can persist in the liver of infected patients and serves as the template to initiate HBV replication. Thus, cccDNA is an important factor for HBV persistence and the recurrence of HBV infection in resolved patients. To study the role of IFN-α in HBV cccDNA transcription, Belloni *et al*[27] utilized a transfection system using the monomeric linear full length HBV genome to show that IFN-α suppresses HBV cccDNA transcription. The HBV interferon-sensitive response element (ISRE) segment mediates STAT1 and STAT2 recruitment on the cccDNA and transcriptional repression by IFN-α. The recruitment of HDAC2, Sirt1, Ezh2, and YY1 to HBV cccDNA was increased by IFN-α treatment, indicating the epigenetic control of HBV cccDNA minichromosome. However, future studies are needed to determine whether and how IFN-α influences the stability of HBV cccDNA and thereby controls HBV replication, as indicated in the clinical studies[28]. The acetylation

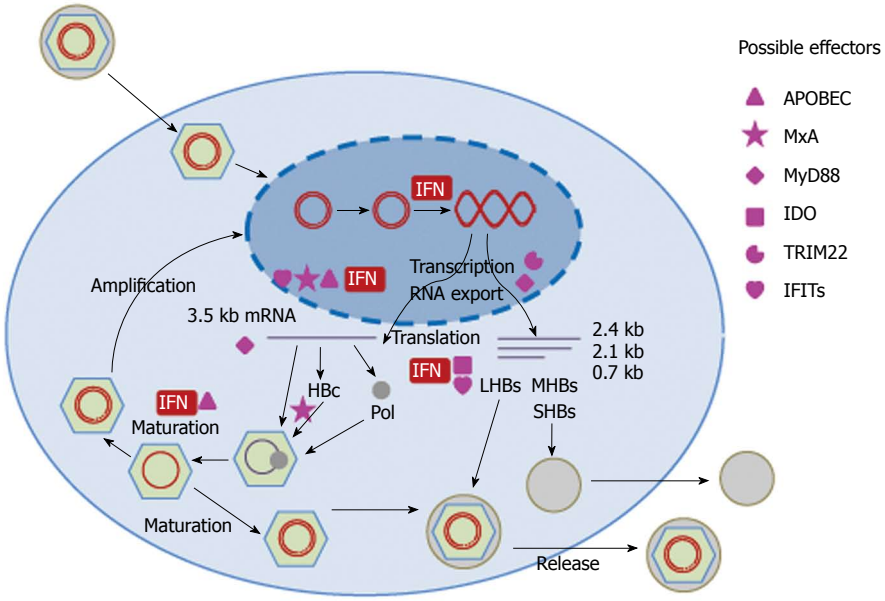

Figure 1 Hepatitis B virus life cycle and the control of hepatitis B virus replication by interferons and interferon stimulated genes. The hepatitis B virus (HBV) life cycle is described in detail in the section 2. The figure summarizes the mechanisms underlying the interferon (IFN)-mediated anti-HBV actions. IFNs have been reported to target multiple steps of HBV life cycle including the epigenetic modification of cccDNA minichromosomes, the enhancer or promoter activity, the RNA stability, and the nucleocapsid formation. Several IFN-stimulated genes (ISGs) represent the effector molecules downstream of IFN signaling: Apolipoprotein B mRNA editing enzyme (APOBEC) family members interfere with HBV capsid formation and maturation and regulate HBV promoter and enhancer activity; MxA affects the HBV RNA export and capsid formation; myeloid differentiation primary response protein 88 (MyD88) was reported to reduce HBV RNA export and the stability of pgRNA; indoleamine 2,3-dioxygenase (IDO) suppresses HBV translation; tripartite motif-containing protein 22 (TRIM22) was reported to control HBV core promoter activity; IFN-induced proteins with tetratricopeptide repeats 1 and 2 (IFITs) limit HBV replication at the transcriptional and posttranscriptional levels.

Table 1 Summary of the anti-hepatitis B virus effects of interferons and interferon stimulated genes

	cccDNA minichromosome	Promoter and enhancer	Posttranscriptional control	Nucleocapsid formation	Others
IFNs	Modifying the composition of HBV minichromosome[27,29] Suppressing cccDNA transcription[27,29] accelerating cccDNA decay[29]	Suppressing HBV EnI and EnII activity[35-37]	Stimulating HBV RNA degradation[42,43]	Inhibiting nucleocapsid formation[48] Accelerating nucleocapsid degradation[49]	
APOBEC	Blocking nucleocapsid maturation[65]	Suppressing HBV S promoter activity[57]			Editing HBV genome[70]
MxA			Inhibiting the nuclear export of HBV RNAs[77]	Blocking HBV nucleocapsid formation[78]	
MyD88			Accelerating the decay of HBV pgRNA[83] Inhibiting the nuclear export of the HBV pre-S/S RNAs[83]		
IDO			Inhibiting translation through tryptophan depletion[88]		
TRIM22		Inhibiting HBV core promoter activity[92]			
IFITs		Inhibiting HBV S promoter activity[96]	Inhibiting HBV replication at posttranscriptional steps[96]		

HBV: Hepatitis B virus; IFN: Interferon; APOBEC: Apolipoprotein B mRNA editing enzyme: catalytic polypeptide; MyD88: Myeloid differentiation primary response protein 88; IDO: Indoleamine 2:3-dioxygenase; TRIM22: Tripartite motif-containing protein 22; IFITs: IFN-induced proteins with tetratricopeptide repeats 1 and 2.

and methylation status of HBV bound histones upon IFN-α treatment needs to be further analyzed. More details about the control of HBV minichromosome by IFNs will be uncovered once the compositions of the HBV cccDNA minichromosome are better defined.

In a recently published study, the antiviral functions of IFN-α on duck HBV (DHBV) cccDNA were analyzed in detail[29]. Base on a chicken hepatoma cell line with tet-inducible DHBV replication, experimental conditions were established where cccDNA was the sole

source for pgRNA transcription and DNA replication. Based on this cell culture system, Guo *et al*[30] demonstrated that IFN-α suppresses cccDNA transcription, which is associated with the reduction of acetylated histone H3 lysines 9 (H3K9) and 27 (H3K27) in cccDNA minichromosome. Their experimental data suggest that IFN-α may induce accelerated cccDNA decay. Although the DHBV cccDNA metabolism and transcription regulation may differ from that of mammalian hepadnaviruses, the study on DHBV cccDNA still provided important clues for HBV cccDNA biology and the antiviral mechanism of IFN-α. Nonetheless, the stability of HBV cccDNA is different *in vivo* and *in vitro*, as hepadnaviral cccDNA decayed within days in cell culture while probably significantly longer in the liver[30-33]. Thus, the mechanism regulating the cccDNA stability should be examined in an *in vivo* model in the future.

IFN-α suppresses the activity of HBV enhancers

Two enhancer elements En Ⅰ and En Ⅱ were identified in the HBV genome and were found to be critical for HBV gene expression and replication[34]. By transient transfection of human hepatoma cells with reporter plasmids under the control of HBV regulatory sequences, several groups have confirmed that type I IFN reduces the activity of En Ⅰ and En Ⅱ[35,36]. Nakao *et al*[37] identified an ISRE-like sequence in the En Ⅰ region and experimentally confirmed that this region could interact with the protein complex containing p48 (ISGF-3γ) and mediate the suppression of En Ⅰ activity by IFN-α. However, the antiviral effect of both IFN-α and p48 was similar in the complete HBV genomes with the wild-type or a mutated ISRE sequence, indicating that the ISRE within the En Ⅰ region is not required for the antiviral effect of IFN-α[38,39]. Despite the lack of functions of the ISRE in the En Ⅰ region in the cell culture, it is not excluded that the interaction between ISGF3 and IFN responsive factors with the ISRE in En Ⅰ could modulate HBV replication *in vivo*. By deletion/mutation analysis, two segments nt 1703-1727 and 1746-1770 within the En Ⅱ sequence were identified to be responsible for the suppressive effects of IFN-α[36]. Evidence shows that IFN-α suppresses the EN Ⅱ activity in a PKC-dependent way. However, the exact function of these segments in the context of replication-competent HBV genomes remains to be uncovered.

Posttranscriptional control of HBV replication by IFNs

A large body of publications indicated that IFNs control the HBV replication at the posttranscriptional level. Chisari's group demonstrated that the secretion of IFN-γ and TNF-α by cytotoxic T lymphocytes (CTLs) could inhibit HBV replication by a posttranscriptional mechanism[8,40]. This could explain the noncytopathic inhibition of HBV replication by CTLs and the reduction of HBV replication in HBV transgenic mice during lymphocytic choriomeningitis virus (LCMV) and murine cytomegalovirus (MCMV) infections[7,41]. Three cellular proteins (p45, p39, and p26) were identified to bind HBV RNAs in association with IFN-γ and TNF-α-induced down-regulation of HBV RNAs[42]. These three proteins were the full length or cleaved products of La protein, a well-described RNA-binding protein, whose membrane expression could be induced by IFN-γ and TNF-α. La protein could bind to the segment nt 1275-1291 of HBV RNA sequence and may contribute to HBV RNA stability[43]. The association between La protein and HBV RNA was further demonstrated by co-precipitation of HBV RNA with human La protein in human hepatoma cells. The modulation of HBV RNA stability by La protein was implied as the half life time of HBV RNA with mutations in the La binding site was reduced[44]. These findings provided evidence for the posttranscriptional control of HBV by IFN-γ and TNF-α through the disruption of La protein that acts as an HBV RNA stabilizing factor. More factors that either stabilize or degrade HBV RNA might also be involved in this process. In fact, the interaction between La protein and HBV RNA is likely modulated by accessory factors in a phosphorylation-dependent manner[44]. Furthermore, the endonucleolytic activity that cleaves HBV RNA near the La protein binding site was upregulated in the liver by CTL injection or MCMV infection.

Inhibition of HBV nucleocapsid formation

The HBV replication in the liver of HBV transgenic mice could be abolished by poly(I:C) injection in an IFN-dependent manner[45]. Further analysis showed that the pgRNA containing capsid in the liver tissue of HBV transgenic mice was eliminated by poly(I:C) injection, either by inhibition of the assembly or acceleration of the degradation[46]. A similar conclusion was reached by experiments with immortalized HBV-transgenic hepatocytes *in vitro*[47]. Later on, IFNs were shown to be responsible to reduced HBV pgRNA containing capsids by the inhibition of HBV capsid assembly[48]. The inhibition of the capsid formation by IFNs might occur at one or several steps in the process of the virion assembly, such as the core protein dimerization, the interaction between polymerase and HBV pgRNA, the encapsidation of polymerase-pgRNA, and the icosahedral capsid formation.

Another report showed that HBV DNA containing capsid was affected by IFN treatment. Xu *et al*[49] explored a cell system derived from immortalized mouse hepatocyte (AML12) with tetracycline (Tet)-inducible transcription of HBV pgRNA and viral DNA replication to examine the antiviral effect of IFN-α and -γ. Compared with Bay-4109 and AT-61, two drugs inhibiting HBV capsid assembly or preventing the incorporation of pgRNA into nucleocapsids, IFN-α and -γ accelerate the decay of HBV DNA containing nucleocapsids in a proteasome-dependent manner. However, the influence of IFNs on the formation of HBV pgRNA containing capsids as described above was not confirmed. This discrepancy probably caused by the different experimental systems and strategies used by the two groups to deter-

mine the influence of IFNs on HBV pgRNA containing capsids. Xu *et al*[49] treated cells with IFN-α and -γ after the establishment of HBV replication, while Wieland *et al*[48] treated cells with IFN-β and -γ prior to the initiation of HBV replication. However, IFNs may interfere with the different steps of HBV capsid formation by activation of the downstream effectors. The ISGs like APOBEC3G and MxA may be involved in the inhibition of HBV nucleocapsid formation as discussed below.

ISGS INVOLVED IN SPECIFIC ANTI-HBV MECHANISMS

Upon binding to the receptors, IFNs activate a variety of IFN-inducible genes through the Janus tyrosine kinase-STAT (JAK-STAT) pathway, which mediate the antiviral effects against HBV[50,51]. Some ISGs are able to trigger common intracellular antiviral pathways. However, the absence of three major antiviral factors IRF1, PKR, and RNase L did not impair the anti-HBV effect of IFN-α/β or -γ[52]. In contrast, the cellular proteasome activity was required for IFN-mediated inhibition of HBV replication[53]. The inducible nitric oxide synthase was also required for the anti-HBV effect of IFN-γ in HBV transgenic mice[54]. To identify the genes linked to the anti-HBV effect of IFN-α/β and -γ, hepatic gene expression profiles were compared in HBV transgenic mice before and after the IFN treatment. Twenty-nine genes were identified and supposed to be associated with the IFN-induced inhibition of HBV replication[55]. However, the role of these identified ISGs is still not fully understood.

Other studies focused on ISGs which were reported to possess antiviral activities. We summarize the available information about those ISGs published so far.

APOBEC family

Apolipoprotein B mRNA editing enzyme, catalytic polypeptide (APOBEC) is a family of cytidine deaminases that edit DNA and/or RNA sequences by deaminating a cytidine base and thereby generating a uridine base. At least 11 members of this family were found in humans, including activation-induced cytidine deaminase (AID) and APOBECs 1, 2, 3A, 3B, 3C, 3DE, 3F, 3G, 3H, and 4. APOBECs are IFN-inducible and play an important role in the innate immune response against many viruses. The antiviral activity of the APOBEC family was first found in HIV infection, and then extended to other viruses including HTLV, HCV, HBV, HPV, HSV-1, and EBV (reviewed in[56]). The control of HBV by members of the APOBEC family, such as AID, APOBEC 1, 3B, 3C, 3F, 3G, and 3H[57-62], could be editing-dependent and independent[63]. In the first report extending the antiviral spectrum of APOBEC3G (A3G) to HBV, A3G was supposed to block HBV DNA accumulation in a way independent on its catalytic activity[64]. Nguyen *et al*[65] further confirmed the deamination-independent inhibition of HBV DNA synthesis by A3G and provided evidence that A3G inhibits the very early steps in viral reverse transcription and blocks DNA strand elongation within the nucleocapsid. Thus, A3G is thought to be incorporated into HBV nucleocapsids. It could be demonstrated that the incorporation of A3G into replication-competent HBV nucleocapsids is specifically dependent on both the viral RT and the packaging signal ε[66]. Thus, A3G likely exerts its anti-HBV activity within HBV nucleocapsids and blocks HBV DNA synthesis and the maturation of viral particles. Another member of the family, APOBEC3B (A3B), was reported to inhibit the binding of hnRNP K (ribonucleoprotein K), a positive regulator of HBV gene expression, to the Enh Ⅱ and to directly suppress HBV-S gene promoter activity[57]. Though the deaminase activity of APOBECs may not represent the main mode of the anti-HBV action since the frequency of edited genomes in cell culture supernatants or patient sera is low[64,67-69], it is unknown whether the majority of edited HBV genomes are able to be secreted. Recent data suggested APOBEC3G (A3G) as the dominant deaminase restricting HBV *in vivo* with up to 35% of HBV genomes being edited[70]. All APOBEC family members except A3DE were able to edit the HBV genome *in vitro* at levels from 10^{-2} to10^{-5} based on the assessment by the 3DPCR technology[71], implying the potential significance of the deaminase activity of the APOBEC family in HBV restriction.

Most of the mutations *in vivo* caused by A3G editing were deleterious, however, a small fraction of genomes could survive and might promote viral evolution and eventually viral evasion of host immune responses. A3G editing might be responsible for the G1896A and G1897A mutations in the HBV precore region which result in the loss of HBsAg synthesis, and for the HBsAg mutations G145E and G145R which are the well-known vaccine escape mutations[70]. It was also suggested that a subset of A3G-edited genomes can be repaired *in vivo*. Kitamura *et al*[72] pointed out that uracil DNA glycosylase could initiate base excision repair of cccDNA and counteract A3G-induced hypermutation of HBV genomes. Though the APOBEC family plays a role in the control of HBV replication, its relative contribution in the IFN-mediated anti-HBV action is still a subject of debate[61,73,74].

MxA

MxA is an interferon-induced dynamin-like GTPase, which has antiviral activity against a wide range of RNA viruses and some DNA viruses including HBV. Though the interaction with viral nucleoprotein is the most likely common mechanism of the antiviral function of MxA[75], its anti-HBV effect might be achieved through more than one pathway. The anti-HBV effect of MxA was firstly demonstrated by Dremsdorf's group by using a Huh7 cell line stably expressing MxA and HBV/MxA transgenic female mice lacking a functional IFN-α/β receptor[76,77]. The MxA expression led to the inhibition of HBV replication and gene expression. MxA inhibits the nuclear export of viral RNAs through the HBV PRE sequence

in Huh7 cells but not in the transgenic mouse model, indicating that MxA exerts the antiviral effect through different mechanisms *in vitro* and *in vivo*[76,77]. The anti-HBV effect of MxA was also verified in HepG2.2.15 cells[78]. Using coimmunoprecipitation and the fluorescence resonance energy transfer (FRET) technique, the authors provide evidence for the direct interaction between MxA and HBcAg. The interaction with MxA causes the immobilization of HBcAg in the perinuclear structures and subsequently the loss of capsid assembly[78]. Thus, MxA is another candidate molecule blocking HBV nucleocapsid formation.

On the other side, HBV developed specific mechanisms to counteract the antiviral activity of MxA. The induction of MxA by IFN-α was impaired in PBMCs isolated from chronic hepatitis B patients, compared with healthy donors[79]. Rosmorduc *et al*[80] showed that HBcAg inhibits the induction of MxA expression. Further investigation confirmed these results and identified the interaction of precore/core proteins with MxA promoter, which was responsible for the downregulation of IFN-induced MxA expression[81].

Myeloid differentiation primary response protein 88

Myeloid differentiation primary response protein 88 (MyD88) is an important molecule in the signaling cascade of the innate immune response mediated by Toll-like receptors (TLRs). Besides, it could be induced by IFNs and is involved in the anti-HBV effect of IFNs. HBV replication is reduced in MyD88-expressing cells and MyD88 overexpression inhibits HBV replication in hepatoma cells HepG2.2.15 and the mouse model[82,83]. MyD88 accelerates the decay of HBV pgRNA and inhibits the nuclear export of the HBV pre-S/S RNAs *via* the posttranscriptional regulatory element[83]. Though the inhibition of HBV replication by MyD88 is evident, the induction of MyD88 upon IFN stimulation is only moderate and not comparable with other ISGs such as MxA. Therefore, its role in the IFN-mediated anti-HBV effect might be limited. On the other side, HBV polymerase blocks the IFN-induced MyD88 expression by preventing nuclear translocation of Stat1 and thereby reducing the activity of the MyD88 promoter[84].

Other ISGs

Indoleamine 2,3-dioxygenase (IDO) is an essential enzyme for tryptophan catabolism, which could cause the tryptophan depletion and suppress adaptive immune response[85]. IDO expression was increased in hepatocytes of HBV transgenic mice after adoptive transfer of HBV-specific CTLs, indicating that HBV infection facilitates the induction of IDO in response to proinflammatory cytokines, particularly IFN-γ[86]. Furthermore, IDO was reported to play a role in the immune tolerance in patients with chronic hepatitis B[87]. Recently, Mao *et al*[88] examined the role of IDO in HBV replication and found that IDO overexpression in HepG2 cells could suppress HBV replication through tryptophan depletion and is likely to be a major factor mediating the anti-HBV activity of IFN-γ since supplementation of tryptophan restores HBV replication inhibited by IFN-γ. Since the conclusion was drawn on the basis of experiments in HepG2 cells, experimental data from *in vivo* models are needed to confirm the role of IDO in IFN-γ mediated anti-HBV effect.

TRIM22 belongs to the tripartite motif (TRIM) family, is inducible by IFNs and has been reported to possess anti-viral activity against HIV, encephalomyocarditis virus (EMCV), HBV, and influenza A virus (IAV)[89-92]. The antiviral effect of TRIM22 had been demonstrated in HepG2 cells as well as in the mouse model and was supposed to inhibit HBV replication through the inhibition of HBV core promoter activity[92]. Previously, Mao *et al*[88] did not observe anti-HBV effect of TRIM22 during the screening of ISGs for their ability to inhibit HBV replication. The discrepancy between these studies is currently not known and needs further clarification.

The IFN-induced proteins with tetratricopeptide repeats 1 and 2 (IFIT1 and IFIT2) are related genes which can be strongly induced by type I IFN. IFIT1 and IFIT2 suppress cellular translation by binding to the translation initiator eIF3 subunits[93,94] and were shown to block viral replication by the sequestration of ppp-RNA (5' triphosphate RNA)[95]. IFIT1 and IFIT2 were identified in a siRNA screening approach to block HBV replication. It was supposed that the baseline expression of IFIT1 and IFIT2 restricts HBV replication at both the transcriptional and posttranscriptional steps[96]. The majority of ISGs with anti-HBV effects were identified by overexpression strategy. We found a drastic enhancing effect of siRNAs targeting IFIT1 and IFIT2 on the HBV replication, though IFIT1 and IFIT2 were only expressed at a relatively low level without IFN-α stimulation. Thus, the baseline expression of ISGs might already contribute to the restriction of HBV replication. This fact probably explains the discrepancy of the anti-HBV effect of the same molecule in different cell lines and in the experiments of different groups. An increasing number of ISGs are identified as restriction factors for HBV replication. However, their relative contribution to the IFN-mediated anti-HBV action remains to be determined. Likely, the anti-HBV function of IFNs is a sum of different ISG functions at different stages of HBV life cycle.

TLR ACTIVATION INHIBITS HBV REPLICATION

Though HBV itself does only trigger innate immune responses to a limited extent, the activation of TLR signaling inhibits HBV replication and is considered a novel therapeutic strategy for the treatment of chronic HBV infection (reviewed in[97]). The ligands specific for TLR2, TLR3, TLR4, TLR5, TLR7, and TLR9 had been examined for their ability to control HBV replication in HBV transgenic mice[98]. All the ligands except for TLR2 could inhibit HBV replication in the liver of HBV transgenic

Table 2 Control of hepatitis B virus replication by Toll-like receptor activation

	HBV transgenic mice[98]	Chimpazee[99]	Hepatoma cell line[100]	Nonparenchymal liver cells[104]	Macrophages[105]
TLR1/2	PGN and Pam3Cys, (-)		LTA , Pam2CSK4[102,103], Pam3CSK4[103], (+)	Pam3CSK4, (-)	Unpublished data, (+)
TLR3	Poly(I:C), (+)		Poly (I:C), (+)	Poly (I:C), (+)	Unpublished data, (+)
TLR4	LPS, (+)		LPS, (+)	LPS, (+)	Unpublished data, (+)
TLR5	Flagellin,(+)			Flagellin, (-)	Unpublished data, (+)
TLR7	R848, (+)		R848, (+)	Single-stranded RNA 40, (-)	Unpublished data, (+)
TLR9	CpG oligodeoxynucleotides, (+)	GS-9620, (+)	cpG DNA, (+)	CpG oligonucleotides, (-)	Unpublished data, (+)

The ligands of different Toll-like receptors (TLRs) used in different experiment systems were shown. Their effect on hepatitis B virus (HBV) replication was presented by "+" which means inhibitory effect or "-" which means no inhibitory effect.

mice in an IFN-dependent manner. Recently, GS-9620, an agonist of TLR7, was administered in chimpanzees with chronic HBV infection and effectively decreased HBV DNA loads in the serum and liver[99].

The antiviral effect of TLR ligands might be mediated by the activation of TLR signaling either in hepatocytes, nonparenchymal liver cells, or extra-hepatic macrophages and dendritic cells, or by the modulation of the adaptive immune response. In hepatoma cells (HepG2), ligands of TLR2, TLR3, TLR4, TLR7, and TLR9 were reported to suppress HBV replication[100]. In the same line, the overexpression of adaptor protein TRIF and IPS, as well as MyD88 mentioned above, could inhibit HBV replication[98,101]. Other groups also showed that stimulation of human hepatoma cells with TLR2 ligands inhibits the HBV DNA replication and nucleocapsid formation[102,103]. For the role of nonparenchymal liver cells in the anti-HBV effect of the TLR ligands, Wu *et al*[104] showed that the local innate immune system of the liver such as Kupffer cells (KCs) and sinusoidal endothelial cells (LSECs) is the mediator of anti-HBV activity of TLR agonists. Guo's group[105] reported inhibition of HBV replication in a murine hepatocyte cell line (AML-12HBV10) by conditioned media from a murine macrophage cell line treated with ligands to TLR1/2, TLR3, TLR4, TLR5, TLR7, or TLR9, indicating a potential role of extra-hepatic macrophages in TLR ligand-triggered anti-HBV effects. Lately, we demonstrated that pretreatment of LSECs with TLR1/2 ligand stimulates the maturation of antigen-presenting LSECs and enable them to activate virus-specific CD8+ T cells[106]. TLR ligands do not only activate the innate immune response but are also involved in modulating the adaptive immune response. Besides the innate and adaptive immune responses, TLR stimulation also modulates the expression of various microRNAs (miRNAs)[107,108]. Since miRNAs were shown to control HBV replication either through direct binding or through indirect mechanisms[109-111], the control of HBV replication by TLR ligands might also be partly achieved through miRNAs (Table 2).

HBV INHIBITS PRR AND IFN SIGNAL TRANSDUCTION

HBV was thought to be a stealth virus; nonetheless, the lack of PRR activation in the liver could be due to the inhibition of PRR and IFN signaling by HBV. Clinical evidence suggests that the expression of TLR2, TLR3, TLR4, TLR7, and TLR9 in PBMCs was reduced in chronically HBV infected patients[112-116]. The reduction of TLR expression might be induced by HBV infection or a result of the exhaustion of TLR system under continuous activation. TLR signaling could be blocked by HBV components through different mechanisms. First, the activation of TLR signaling might be blocked by HBV infection since the activity of TBK1/IKKε, a key molecule downstream of the TLR signaling pathway, was inhibited by HBV pol through disrupting the interaction between IKKε and DDX3[117]. Further, recent findings suggest that the activation of TLR signaling in nonparenchymal liver cells, extra-hepatic macrophages, and dendritic cells is inhibited by HBV. Wu *et al*[118] found that pretreatment of hepatocytes and nonparenchymal liver cells with HBsAg, HBeAg, or HBV virions significantly reduced the TLR-induced antiviral activity, and this was correlated with the suppressed IRF3, NF-κB and ERL1/2 activation in pretreated cells. The response of pDC to TLR9 ligand was reduced by HBV through the blockage of the MyD88-IRAK4 axis[113], or through the HBsAg-mediated upregulation of SOCS-1 (suppressor of cytokine signaling-1) expression and BDCA-2 (dendritic cell lectin B) ligation[119]. Wang *et al*[120] reported that HBsAg inhibits TLR2 ligand (Pam3csk4)-induced JNK activation and IL-12 production in monocytes/macrophages.

Above, we discussed the role of ISGs in the control of HBV replication. However, HBV is able to evade the functions of ISGs by counteracting IFN signaling. HBV pol protein was reported to inhibit TBK1/IKKε activity by disrupting the interaction between IKKε and DDX3[117]. The downstream of JAK-STAT signaling pathway activated by IFNs which resulted in the induction of ISGs could be blocked by HBV. As discussed above, HBV pol inhibited STAT1 translocation in HepG2 cells and thus impaired the IFN-induced MyD88 promoter activity[84]. The response of HBV-infected human hepatocytes in chimeric mice was also reduced compared to that of uninfected human hepatocytes, as shown by the reduced STAT-1 nuclear accumulation[121]. Recently, Chen *et al*[122] explored the mechanism by which HBV impairs IFN-induced STAT1 translocation. They demon-

strated that HBV pol suppresses IFN-α–induced STAT1 serine 727 phosphorylation through the interaction with the catalytic domain of protein kinase C-δ (PKC-δ). HBV pol protein interferes also with nuclear transportation of STAT1/2 by binding to importin-α5[122].

HBV GENOME MUTATION AND IFN RESPONSE

HBV genome mutation might be one factor determining the response to IFN treatment. *In vitro* studies in hepatoma cell lines showed a lower sensitivity of HBV with precore or BCP mutations to IFN-α[123,124]. In a recent clinical investigation[125], 214 HBeAg-positive CHB (Chronic hepatitis B) patients were recruited and treated with PEG-IFN ± lamivudine for 52 wk, and the relationship between the response to IFN treatment and the presence of wild type or non-WT were calculated. It was concluded that the presence of only WT virus at baseline is a strong predictor of response (HBeAg loss with HBV DNA < 10000 copies/mL) to PEG-IFN for HBeAg-positive CHB. Patients with detectable PC (precore) and/or BCP (basal core promoter) mutants have a lower probability of response and are less optimal candidates for PEG-IFN therapy. The same cohort was followed to 78 wk. The relationship between HBsAg levels and response to IFN depends upon the presence of PC/BCP mutations[126]. Besides, the mutation in HBV ISRE was supposed to influence IFN response in CHB patients[127]. On the other hand, HBV variants can be selected during IFN-α therapy[128].

CONCLUSION

It is well accepted that activation of TLR and the production of IFNs contribute to the control of HBV replication. Due to the complexity of TLR and IFN signaling and functions, the antiviral actions of these pathways are not completely understood. The stimulation of IFN and TLR signaling pathways may be explored as therapeutic approaches for chronic viral infections. Recently, several preclinical studies were presented to show the potential usefulness of TLR ligands (*e.g.*, TLR7) for the treatment of chronic HBV infection. Furthermore, the identification of the effector molecules downstream of IFN and TLR signaling pathways, especially those involved in HBV cccDNA elimination, will provide more options for the development of anti-HBV drugs.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (9): Hepatitis B virus

Molecular mechanism of hepatitis B virus-induced hepatocarcinogenesis

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Abstract

Hepatitis B virus (HBV) infection is a global public health problem with approximately 2 billion people that have been exposed to the virus. HBV is a member of a family of small, enveloped DNA viruses called hepadnaviruses, and has a preferential tropism for hepatocytes of mammals and birds. Epidemiological studies have proved a strong correlation between chronic hepatitis B virus infection and the development of hepatocellular carcinoma (HCC). HCC is the fifth most common malignancy with about 700000 new cases each year, and more than 50% of them arise in HBV carriers. A large number of studies describe the way in which HBV can contribute to HCC development. Multiple mechanisms have been proposed, including the accumulation of genetic damage due to immune-mediated hepatic inflammation and the induction of oxidative stress. There is evidence of the direct effects of the viral proteins HBx and HBs on the cell biology. Integration of HBV-DNA into the human genome is considered an early event in the carcinogenic process and can induce, through insertional mutagenesis, the alteration of gene expression and chromosomal instability. HBV has also epigenetic effects through the modification of the genomic methylation status. Furthermore, the virus plays an important role in the regulation of microRNA expression. This review will summarize the many mechanisms involved in HBV-related liver carcinogenesis.

Key words: Hepatocellular carcinoma; Hepatocarcinogenesis; Hepatitis B Virus; Chronic hepatitis B infection; Cell biology

Core tip: Hepatitis B virus (HBV) infection is a global health problem. There is evidence that HBV have a causal role in the development of hepatocellular cancer, but the mechanism leading to the transformation of normal hepatocytes into cancer cells is still intricate. This review will summarize the many mechanisms involved in HBV-related liver carcinogenesis.

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HEPATOCELLULAR CARCINOMA

Hepatocellular carcinoma is a highly prevalent and lethal neoplasia. It is the fifth most common cancer in men and the ninth in women with respectively about 500000 and 200000 new cases per year; looking in detail the major number comes from Asia (76%), followed by Europe

(8.1%), Africa (7.5%), North America (4.2%), Latin America and Caribbean (3.8%) and Oceania (0.4%)[1]. The global distribution of these new cases is uneven and reflects the differences in the exposition of the local populations to the different etiological factors: in Eastern Asia and Sub-Saharan Africa the dominant risk factor is chronic HBV infection, while in North America, Europe and Japan HCV infection together with excessive alcohol intake are the main risk factor. Furthermore in developed countries the increasing incidence of obesity and consequently of non-alcoholic steatohepatitis is becoming an important risk factor for cirrhosis and HCC as well[2,3]. Other risk factors include type 2 diabetes, hemochromatosis, Wilson`s disease, alpha-1 antitrypsin deficiency, glycogen-storage diseases, autoimmune hepatitis, tyrosinemia and some porphyries[4-6]. Independently from the etiologic factor underlining the presence of chronic liver disease, cirrhosis should be consider major risk factor, in fact 70%-90% of HCC develop from these group of patients.

Even if in the last decades the management of HCC is improved due to the increase diagnostic capacity, the development of evidence-based staging system and the availability of new effective treatments the prognosis is still very poor (overall ratio of mortality to incidence of 0.95)[1] suggesting that a deeper comprehension of the molecular pathway involved in the hepatocarcinogenic process is necessary to improve the outcome of HCC patients.

HBV BACKGROUND

Hepatitis B virus (HBV) infection is a major global public health problem with approximately 2 billion people that present evidence of contact with the virus. Considering that more than 350 million individuals in the global population are chronic HBV carriers, this virus stands as one of the most common human pathogens[7]. The risk of developing chronic hepatitis B (CHB) infection after an exposure seems to depend on the age at which the virus is first contracted[8]. Infection within the first year of life has a 90% chance in developing a CHB; infections in childhood represent a risk of 20%-30%; less than 1% of the exposure in adults go on to develop CHB[8]. Chronic infection may progress to cirrhosis and ultimately hepatocellular carcinoma (HCC). CHB infection remains the major etiological factor of HCC worldwide with more than one half of HCC patients being HBV infected[9]. Several observations indicate an etiologic association between CHB and the development of HCC including a high prevalence of HBV surface antigen (HBsAg) among HCC patients. CHB increases up to a 20-fold the risk of developing HCC also in the absence of cirrhosis. The REVEAL-HBV study found that serum HBV DNA levels and HCC risk correlate in a linear relationship[10]. Furthermore the presence of HBV-DNA integration within the hepatic cells increases about 100 times the relative risk for HCC among HBsAg carriers compared with negative individuals[11].

Specific viral and host factors can contribute to an increased risk of HCC among patients with CHB: increasing age, male gender, and longer duration of infection, all increase the risk of HCC and the presence of cirrhosis is the single most-important risk factor for HCC[12].

Host genetic background is known to have an influence in the story of the disease; in fact belonging to a specific ethnical group increases the susceptibility of HBV carrier to develop HCC. Among all Africans have the worst outcome, followed by Asians[13]. In these two geographic areas more than 70% of HCC develop in patients with HBV infection[14]. In Asia annual incidence among HBV carriers is more than 0.2% with a risk of HCC development before the cirrhotic stage[15]. In Africa of great importance is the potential aflatoxin exposure that increases highly the risk, obliging to start HCC surveillance in youth. The synergic effect of HBV infection and aflatoxin exposure increases the risk to develop HCC by 60 time compared to healthy individuals[16].

Several meta-analysis tried to associate genetic polymorphisms with an increased risk of HCC in CHB carriers; contradictory evidences are present in literature. While more studies are needed to unveil the mechanisms connecting some of these genetic changes to HCC, current results suggest a positive correlation of TNFα and GSTT1 polymorphisms with HCC[17]. On the viral side, for a long time the attention has been focused on high viral load as predictive for HCC development, but other viral risk factors have been found including the viral genotype. Until now 11 HBV genotypes (A–J) have been identified, based on differences in their genome sequence[18]. HBV genotypes have distinct geographical and ethnic distributions: genotype A is pandemic but most prevalent in northern Europe, North America and central Africa; genotypes B and C are found in eastern Asia, Korea, China, Japan, Polynesia and Vietnam; genotype D is also pandemic but is predominant in the Mediterranean area, the Middle East and India; genotype E is typical for Africa; genotype F is found in Native Americans and in Polynesia; genotype G is present in western Europe and North America and genotype H is found predominantly in Central America. The epidemiological evidences indicate that the genotype C has a higher risk of causing HCC than B, and D has a higher than A[19]. Furthermore the presence of enhancer II/basal core promoter mutations (A1762T/G1764A), and mutants with preS deletions are associated as well with increased risk of HCC[20,21]. External cofactors that can also promote HCC development in CHB carriers are concomitant infection with human immunodeficiency virus (HIV), hepatitis C or D virus, cigarette smoking, environmental pollution, chronic alcohol consumption, aflatoxin exposition, and metabolic syndrome[12,17,22]. HCC is the third leading cause of cancer-related death worldwide[23]. Recent epidemiological data have demonstrated that liver cancer incidence is continuously rising and will continue to do so for more than a decade, not only in Asia and Africa but also in North America and Europe[9]. Despite the avail-

ability of an efficacious and safe hepatitis B vaccine[24] about 600000 people die worldwide every year due to either the acute or chronic effects of the virus, with a high proportion dying of HCC. In this context, advances in our understanding of the molecular basis of HCC are urgently needed to develop early tumour markers and novel targeted agents with improved therapeutic efficiency[25]. Here we review the molecular mechanisms linking CHB to malignant transformation of liver cells.

HEPATITIS B VIRUS

HBV is a member of a family of small enveloped DNA viruses called hepadnaviruses, which infect a restricted number of mammals and birds. These viruses share a narrow host range and preferential tropism for hepatocytes. The genome of hepadnaviruses is a circular, partially double-stranded DNA genome that replicates via an RNA intermediate[26]. The HBV genome consists of two asymmetric DNA strands, which forms a partially double-stranded relaxed circular DNA structure that is around 3.2 kb in length. The HBV genome is organized into 4 overlapping open-reading frames (ORFs) that give rise to 5 messenger RNAs (mRNA). The largest one is a 3.5 kb mRNA strand that is composed of two subspecies: a 3.5 kb precore/core mRNA that is translated into the e antigen (HBeAg), and a second 3.5 kb strand termed the pregenomic mRNA that is translated into the core and polymerase proteins. The remaining mRNAs include the 2.4 kb large and 2.1 kb small surface mRNAs, which encode the three viral surface proteins. The 0.7 kb small mRNA gives rise to the hepatitis B X (HBx) protein that is essential for virus replication and appears to play an important role in HBV-induced HCC. The HBV genome encodes also a 2.2 kb singly spliced pre-genomic RNA producing the newly discovered hepatitis B spliced protein (HBSP) involved in proliferation and viability of HBV-infected cells[27,28].

The HBV enters the hepatocytes and releases, by disintegration of the nucleocapsid, the relaxed circular DNA (rcDNA) that can be transported to the nucleus, where it is converted into covalently closed circular DNA (cccDNA). It is present in the nucleus of infected hepatocytes bound to both histone and nonhistone proteins, in a stable freestanding episomal conformation. cccDNA serves as the template for transcription of all viral mRNAs and is unaffected by all current nucleotide analog antivirals because they inhibit DNA replication, which occurs after cccDNA formation. In fact cccDNA persists during therapy even after the clearance of HBsAg, and this is the reason why disease recurrence is possible even after successful treatment[29,30].

MECHANISMS OF HBV-RELATED HCC INDUCTION

HBV can promote HCC in many ways. There is a large amount of data describing the multiple pathways involved in this process, including the accumulation of genetic damage due to immune-mediated hepatic inflammation, the induction of oxidative stress, a virus-specific mechanisms involving the viral proteins HBx and HBs, the insertional mutagenesis with integration of HBV DNA into the host genome that alters the expression of endogenous genes or induces chromosomal instability, epigenetic modification through the modification of the genomic methylation status and also the regulation of microRNA (miRNA) expression.

Immune and inflammatory factors

In many different tissues chronic inflammation is known to play a vital role in cancer development. In the liver, repeated cycles of inflammation induced apoptosis and hepatocyte regeneration, increasing the risk of hepatocarcinogenesis. T cell dysfunction, cytokine production, and inflammation-mediated alteration of specific signaling pathways play important role in the development of HCC. During inflammation, the activation and interaction between STAT3 and nuclear factor (NF)-κB play vital roles in controlling the communication between cancer cells and inflammatory cells. NF-κB and STAT3 are two major factors that keep in check the ability of preneoplastic and malignant cells to resist apoptosis-based tumor-surveillance and regulate tumor angiogenesis and invasiveness. HBV infection and inflammation induced NF-κB activation, that in turn promoted immune escapes, facilitating the development of HCC. STAT3 activation induced by interleukin (IL)-6, IL-6 cytokine family, and IL-22, also promoting the development of HCC[31,32].

In the immune system, $CD4^+$Th1 and $CD8^+$ T cells play an important role in the growth inhibition and the death of cancer cells. This ability seems to be related to their capability to secrete INF-γ, but other cytokines may be involved; in several experimental studies have been proved that the depletion of these population of T cells impaired the immune response against the cancers and the rejection of the implanted tumor cells suggesting theirs involvement in the immune response against cancer[33]. Differently the regulatory T cells (Treg) are a subpopulation of T cells ($CD4^+$, $CD25^+$and $Foxp3^+$) which maintain tolerance to self-antigens, and abrogate autoimmune disease by suppressing immune responses of other cells. Treg cells have been reported to play a key role in the immune impairment involved in HBV-related HCC. Elevated TGF-β activity, associated with the persistent presence of HBV in the liver tissue, suppresses the expression of miRNA-34a, leading to enhanced production of chemokine CCL22, which recruits Treg cells[34]. HBV infection was found to increase the immunomodulatory activity of Treg by up-regulating the expression of forehead box P3 transcriptional regulator (FoxP3), cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) and glucocorticoid-induced tumor necrosis factor (TNF) receptor family gene (GITR). The expansion of Treg cells and the enhancement of their suppressor function prevent the anti-tumor immune response against HCC tumor antigens and inhibit tumor immune surveillance against HCC[35]. Finally, Li *et al*[36] were able to investigate Treg

cells in the circulatory blood and tumor tissues of patients with HBV-related HCC and their data indicate that an increased amount of Treg cells either in peripheral blood or in the tumor tissue correlates to poor prognosis.

HBV and oxidative stress

In literature, there are many indications that HBV can induce a pro-oxidative status. In fact, increased level of oxidative stress, sulfhydryl and lipid peroxidation were found in CHB patients[37]. Additionally, typical products of reactive oxygen species (ROS) are enhanced in carriers with high HBV DNA titers[38].

Oxidative stress is a disturbance in the cellular equilibrium that can result from a lack of antioxidant deference capacity or by an increased production of ROS. The excess of ROS can damage lipids, proteins or DNA, and consequently alter different cellular pathways and influence gene expression, cell adhesion, cell metabolism, cell cycle and cell death. ROS-induced oxidative DNA damage can increase chromosomal aberrations associated with cell transformation[39]. ROS may also activate cellular signaling pathways, such as those mediated by mitogen-activated protein kinase (MAPK), NF-κB, phosphatidylinositol 3-kinase (PI3K), p53, FOXO, β-catenin/Wnt, COUTFII and others associated with angiogenesis[40-42].

Because of the role of these pathways in mutagenesis, tumor promotion, and progression, ROS are considered potential carcinogens[43].

In vivo and *in vitro* experiments have shown that HBV infection is able to induce oxidative stress by reproducing the same increase found in CHB patients. Furthermore, to demonstrate that oxidative stress plays a critical role in hepatic injury, several studies showed that the total peroxide levels, a parameter of oxidative stress, as well as alanine aminotransferase (ALT) levels are significantly higher in patients with chronic hepatitis compared to asymptomatic carriers[44].

Interestingly, transgenic mice that produce and accumulate HBsAg inside the hepatocytes have increased levels of inflammation and oxidative stress before the development of dysplastic foci and HCC[45,46]. Moreover, transgenic mice that express HBx protein[47] have high levels of ROS, suggesting that the viral induction of oxidative stress occurs through many mechanisms.

Mitochondria are a major source of ROS inside the cells, which can be produced through electron leakage from the mitochondrial respiratory chain[48]. HBx targets mitochondria binding to the voltage-dependent anion-selective channel protein 3 (VDAC3), and alters the mitochondrial membrane potential and increases endogenous ROS levels[49-51]. HBx also induces oxidative stress through cytosolic calcium signaling, resulting in Ca++ accumulation into mitochondria, consequent increased levels of ROS and activation of cellular kinases (PYK2 and SRC kinases), leading to the activation of transcription factors NF-κB and STAT3 which promote HBV replication and early steps of HCC[52-54]. The increased level of Ca++ in presence of ROS can trigger endoplasmic reticulum (ER) stress and the unfolded protein response (UPR). In fact, if the ER stress remains constantly elevated, UPR signaling cannot be maintained and the cell activates the autophagic process to restore ER integrity, which is important for viral replication[55]. HBx alone in the context of whole viral genome transfections caused the mitochondrial translocation of mitogen-activated protein kinase Raf-1. This event induced by oxidative stress involves the Src- and the PAK-mediated phosphorylation of Raf-1, leading to its activation[56]. HBx also influences the lipid peroxidation *via* downregulation of SeP expression, resulting in increased expression of TNF-α as shown in an *in vitro* study in the human hepatoblastoma cell line HepG2[57].

Other HCC cell lines (human HuH7 and murine ML1-4a), stably transfected with the pre-S mutants (truncated forms of preS/S polypeptide), exhibited enhanced levels of ROS through endoplasmic reticulum (ER) stress pathways. The oxidative DNA damage has also been confirmed in the livers of transgenic mice carrying the pre-S mutant[58].

HBV-DNA Integration

Another proposed mechanism of induction of HCC by HBV is through its integration in the host genome. HBV-DNA integration into human host chromosomes occurs in the infected liver since early stages of natural acute infections. Multiple integrations have been detected in chronic hepatitis tissues, and integrated HBV sequences have been seen in 80%-90% of HBV-related HCCs[59]. HBV insertions have been associated with major genetic alterations within the cell genome, including generalized genomic instability, gene and chromosomal deletions and translocations, amplification of cellular DNA, and generation of fusion transcripts[60-61]. These alterations in the host genome may alter the expression of miRNAs, oncogenes, and tumor-suppressor genes, events that could lead to the development of HCC.

Early studies suggested that viral integration into the host gene occurred randomly, but in the last decades with the introduction of whole genome sequencing, several groups were able to identify some preferred site for HBV integration, typically close to or inside of certain target genes, such as TERT (telomerase reverse transcriptase), *FN1* (Fibronectin 1), SMAD5 (SMAD family member 5), *MLL4* (Myeloid/lymphoid or mixed-lineage leukemia 4), ARHGEF12 (Rho guanine nucleotide exchange factor GEF 12), CYP2C8 (Cytochrome P450, family 2, subfamily C, polypeptide 8), PHACTR4 (Phosphatase and actin regulator 4), PLXNA4 (Plexin A4), RBFOX1 (RNA binding protein, fox-1 homolog), ADH1B (Alcohol dehydrogenase 1B), CPS1 (Carbamoyl-phosphate synthetase 1), ESRRG (Estrogen-related receptor gamma), LRFN2 (Leucine rich repeat and fibronectin type III domain containing 2), MYOM1 (Myomesin 1), RAI1 (Retinoic acid induced 1), CTDSPL2 (CTD small phosphatase like 2), LRP1B (Low density lipoprotein-related protein 1B), SENP5 (SUMO1/sentrin specific peptidase 5), ROCK1

(Rho-associated, coiled-coil containing protein kinase 1), PDGF receptor and CCNE1 (cyclin E1)[59,62-64]. The identification of these and other recurrent sites of viral integration inside or in the proximity of genes controlling cellular proliferation, survival, differentiation and immortalization, suggests that this process may indeed be involved directly in hepatocarcinogenesis.

However, evidence for fusion proteins came from a direct promoter insertion mechanism that was first provided in HBV-related HCC where insertion targeted either the retinoic acid receptor-β (RAR-β) gene or the human cyclin A gene, resulting in tumour-specific chimeric proteins endowed with novel, pro-carcinogenic functions.

Finally, although integrated viral sequences are defective for replication, they might also contribute to tumorigenesis through the production of truncated and mutated HBx or preS2/S proteins. These proteins may act on HCC development by disrupting the control of cellular gene expression or by activating oncogenic signaling pathways[65,66].

DNA methylation

DNA methylation is one of the most intensely studied epigenetic modifications in mammals. Aberrant DNA methylation patterns - hypermethylation and hypomethylation compared to normal tissue - have been associated with a large number of human cancers. Hypermethylation typically occurs at CpG islands in the promoter region and is associated with the inactivation of certain tumor-suppressor genes. The enzymes responsible for the maintenance of methylation patterns are the DNA methyltransferases (DNMT) and a large body of evidence shows that HBx upregulates DNMT1, DNMT3A1 and DNMT3A2[67]. Conversely, global hypomethylation has also been reported to induce genomic instability and contribute to cell transformation and progression of cancer.

In HCC, alteration of DNA methylation occurs in the early stage of cancer development, and in these patients the increased risk of cancer development was due both to genomic hypomethylation with related increased chromosome instability and localized hypermethylation with decreased tumor suppressor gene expression[67].

Lately, DNA hypermethylation in the promoter region of specific oncosuppressor genes was found in HBV-related HCC[68-70]: RASSF1A (Ras association domain family member 1), p16INK4A and P21WAF1/CIP1 are involved in cell cycle maintenance and their altered expression is an early event in the development of HCC; CDH1 (E-cadherin) is involved in cell adhension and metastatization; GSTP1 downregulation exposes cells to oxidation damage and electrophilic carcinogens; ASPP1 and ASPP2 have an important role in apoptosis. Alterations were found also in the promoter region of hTERT, maintaining its expression and increasing proliferative capacity of the cell, and in the promoter region of COX-2 leading to a more proinflammatory state.

HBx protein

The viral protein HBx is a 154 amino acids long protein acting as a pleiotropic transactivator; it does not bind directly to DNA but rather acts on cellular promoters by protein-protein interactions and modulating cytoplasmic signaling pathways.

Interestingly HBx is expressed at low levels during acute and chronic hepatitis and can induce a humoral and cellular immune response[71,72]. Thanks to viral DNA integration into the host genome, the HBx gene is maintained and transcribed in human HCC tumor cells even if complete HBV replication is absent[73,74].

In the cytoplasm, it activates mitogenic signaling cascades while in the nucleus it modulates gene expression via interaction with numerous transcription factors. The large body of evidences suggests its central function in a large number of signaling pathways involved in oncogenesis, proliferation, apoptosis, inflammation and immune response. Furthermore, HBx may act as a paracrine factor and activates stellate cells[75,76].

HBx play a role in chromosomal instability by targeting centrosome dynamics and mitotic spindle formation through its binding with different cellular partners implicated in centrosome formation. HBx has been suggested to induce multipolar spindle formation, chromosome segregation defects, and appearance of multinucleate cells by inducing aberrant centrosome duplication; these biological actions might be due to sequestration of the nuclear transport receptor Crm1 in the cytoplasm[77], and/or HBx binding to the hepatitis HBx interacting protein (HBXIP), a regulator of centrosome duplication[78] or to the UV-damaged DNA binding protein 1 (DDB1)[79] and induction of lagging chromosomes by binding to BubR1[80].

HBx may increase the expression of matrix metalloproteinase and facilitated cellular migration[81-83].

HBx transactivates a number of cellular promoters and enhancers containing binding site for NF-κB, activator protein 1 (AP-1), AP-2, CCAAT-enhancer-binding protein (c-EBP), RNA polymerase and nuclear factor of activated T-cells (NF-AT), cellular promoter of genes associated with cell proliferation as IL-8, TNF, transforming growth factor (TGF) beta and epidermal growth factor receptor (EGRF) and cytosolic signal transduction pathways as Ras/Raf mitogen-activated protein kinase, Src kinases, cJun Nterminal kinase, Jak1/STAT and protein kinase (PK)[84,85], which have overlapping effects on cell proliferation and viability.

HBx interacts with the acetyltransferases CBP/p300, and this interaction induces the activation of CREB-dependent transcription. The activation of CREB/ATF trans-activation function by HBx appears redundant since HBx has been shown to increase CREB/ATF DNA-binding affinity as well as to enhance the recruitment of CBP/p300 to CREB/ATF bound to cellular DNA[86,87]. The modulation of CREB/ATF plays an essential role in liver metabolism and proliferation, and CREB has been implicated in hepatocarcinogenesis[88].

Important targets of HBx are p53 and p53 family[89]. In fact, the viral protein directly binds to p53 and impairs its function. HBx, through the interaction with p53, can alter p53-mediated apoptosis, transactivation properties of p53[90], cell cycle regulation[91], DNA repair genes[92,93], and tumor suppressor genes[71].

In the mitochondria HBx interacts with the heat shock protein 60 and 70[94] and the voltage dependent anion channel (VDAC) isoform VDAC3[50].

HBx seems to regulate the angiogenic process in HCC[95-98]. Indeed, HBx expression induces transcriptional up-regulation of the vascular endothelial growth factor (VEGF) and the proangiogenic growth factor angiopoietin 2 (ANG2). It also plays an important role in the hypoxia inducible factor HIF-1 cellular level. In fact HBx binds to and stabilizes HIF1α and at the same time stimulates HIF1α transcription, thus promoting angiogenesis.

HBx can activate Wnt/beta-catenin signaling in two different ways: by up-regulating cytoplasmic beta-catenin[99] or alternatively by hypermethylating E-cadherin promoter and consequently repressing its transcription[100].

HBV preS/S proteins

The preS/S ORF encodes three different, structurally related envelope proteins refered to as the large (L), middle (M), and small (S) proteins that are synthesized from alternative initiation codons. These three proteins share the same carboxy-terminus part but have different amino-terminal extensions. In particular, the S protein corresponding to the HBV surface antigen (HBsAg) consists of only 226 amino acids (aa), the M protein contains an extra N-terminal extension of 55 aa, and the L protein has a further N-terminal sequence of 108-119 aa compared with the M protein. Until now, few mechanisms of action of preS/S encoded proteins have been known to be involved in the hepatocarcinogenic process. During HBV replicative cycle, HBsAg can accumulate into the ER, induce ER stress and consequently increase the cellular level of oxidative stress. This process happens also in presence of pre-S2 mutants in which the viral proteins amass in the ER. The induced ER stress upregulates the cytoplasmic Cyclin A, increasing infected cell proliferation; at the same time, Cyclin A upregulation can promote, through centrosome over-duplication, chromosome instability which is a well-known mechanism in HBV-related hepatocarcinogenesis[101].

Additionally, HBsAg seems to have an effect on the mitochondrial function: on one hand it binds to enoyl coenzyme A hydratase short chain 1 (ECHS1) and can induce cell apoptosis by decreasing the mitochondrial membrane potential (MMP)[102], while on the other hand HBsAg could inhibit JTB (jumping translocation breakpoint), leading to increased cell motility and decreased apoptosis[103]. Interestingly, pre-S2 mutant protein in type Ⅱ ground glass hepatocytes (GGHs) could directly interact with the c-Jun activation domain-binding protein 1 (JAB1), inducing an hyperphosphorylation of the tumor-suppressor retinoblastoma (RB) and its inactivation[104].

Futhermore, pre-S2 protein can act as a transactivator and, interacting with the hTERT promoter[105], increase telomerase activity, a key step in the development of HCC and other cancers. Interestingly, HBV is also able to upregulate of telomerase activity by HBV DNA integration in proximity of the promoter[106] or by HBx capacity to increase the SP1 binding to hTERT promoter and induce its transcription[107]. Despite hTERT activation, telomere in HCC cells remain shorter than in normal somatic cells, predisposing to occasional telomere and chromosomal instability, and polyploidy[108]

miRNAs

MiRNAs are small non-coding RNA molecules (19-25 nucleotides in length) that regulategene expression at transcriptional and post-transcriptional levels, usually resulting in gene silencing via translational repression or target degradation of gene mRNA. Since their discovery in the early 1990s, over 1000 miRNAs have been characterized in human cells. In the last decade, many groups have investigated the role of this biological entity in patho-physiological processes; several publications have unveiled the function of miRNAs in the development, progression and metastatization of HCC. Specifically, a growing number of studies were able to identify specific miRNAs modulated in CHB patients; some of them are regulated by HBV infection and have a role in hepatocarcinogenic process.

miR-143, miR-34, and miR-19 have been found to be upregulated in HBV-related HCC and to promote a more aggressive cancer phenotype, while Let-7a downregulation by HBx increased cell proliferation[109]. HBx also downregulates miR-152 with the consequent upregulation of DNMT1, which methylates the promoters of many tumor suppressor genes. Interestingly, other publications reported additional miRNAs that are involved in HBV-related HCC[110]: miR-221, which is downregulated in acute HBV infection, normally expressed in chronic HBV infection and upregulated in HCC; miR-101, which is constantly downregulated in HBV infection and in HCC tissues, has been associated with HCC development; miR-18a/miR-18b and miR-106a may be key effectors for the progression of HCC. Very recent data suggest other possible miRNAs involved in HCC development in CHB patients: miR-224 that is inversely correlated to autophagy in HBV-related HCC specimens[111], miR-122 that is regulated by HBx through PPARg[112], miR-15a and miR-16-1 directly downregulated by HBx[113], miR-132 regulated by the hypermethylation status induced by HBx[114], or miR-148 that is also downregulated by HBx through the suppression of p53-mediated activation[115]. Recently, Xu *et al*[116] reviewed the HBV-HCC correlation and reported miR-602, miR-143, miR-29a, miR-148a, miR-373, miR-101, miR-152, miR-16, and miR-661 as miRNAs possibly implicated in the carcinogenic process. In the future, more extensive analysis will be able to create a precise profile of HBV modulation of miRNAs and

their function in the different phases of the hepatocarcinogenetic process.

FUTURE THERAPEUTIC STRATEGIES

Up today HCC remain a devastating cancer, so the safest strategy remain the prevention, through the control of the risk factors. Prevention of HBV infection among them can be accomplished by large scale vaccination or in patients with preexistent CHB a continued suppression of HBV replication with antiviral (*e.g.*, entecavir and adefovir dipivoxil) can prevent complications of HBV-related liver disease and decrease the risk of HBV-related HCC development[117]. In fact the REVEAL-HBV study suggests that the degree of HBV viremia predicts HCC risk independently of cirrhosis, HBeAg+ or ALT levels, introducing the concept that virologic suppression through antiviral therapy may have a major impact on the prevention of liver cancer. After the rise of HCC then the treatment selection follows the Barcelona Clinic Liver Cancer (BCLC) staging system that assigns the prognosis and proposes the therapeutic strategies for each stage. In the proposed strategies only one systemic chemotherapeutic molecule (Sorafenib) is approved as first line of treatment. Since the emergence of Sorafenib as the new standard for the systemic treatment of HCC, the idea of targeting specific molecular pathways implicated in the pathogenesis and progression of HCC, has promoted a revolutionary change in the treatment of this disease. Genetic modifications and alteration of critical molecular signaling pathways have been identified as contributing to the tumor development and progression. Among the numerous signaling pathways implicated in the development and growth of HCC are worth to be noted Ras/Raf/MAPK, Wnt-b-catenin, EGFR, insulin-like growth factor receptor, VEGFR, NF-KB, AKT-mTOR, Notch and Hedgehog. Thanks to the acquired knowledge on altered pathways in HCC several promising novel anticancer agents are currently under evaluation (56 drugs)[118] including tyrosine kinase inhibitors, monoclonal antibodies and oligonucleotide antisense. Furthermore monotherapy can be very effective in vitro but targeting one pathway may result in the activation of other pathways in HCC cells suggesting that the most attractive strategy for the future should be the combination of different targeted agents to improve the efficacy of these molecules[119,120]. Research will permit a more comprehensive understanding of hepatocarcinogenic process and to identify new molecular targets for therapeutic intervention.

CONCLUSION

Among the different etiologic agents, HBV is the major risk factor for developing HCC. In the future, the global neonatal vaccination program will greatly reduce the burden of HBV and ultimately of HCC, but more than 700000 new HCC cases are still identified each year. In the last decades, progresses have been made in understanding the multifactorial process through which HBV infection can promote hepatocarcinogenesis. On one hand, HBV infection is able to induce a chronic inflammatory status in the patient, to increase the oxidative stress and to modulate the host immune response against infected hepatocytes. On the other hand, HBV DNA integration may lead to chromosomal instability and alteration of gene expression. Furthermore, HBV products directly disrupt normal cellular signal pathways, contributing to induce HCC. Other factors like genomic methylation and miRNA expression have been identified to play a role in HBV-related development of HCC.

A deeper knowledge of the mechanisms that mediate the HBV carcinogenic process is essential for developing novel strategies to prevent and treat liver cancer in chronic HBV carriers.

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TOPIC HIGHLIGHT

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Management of antiviral drug resistance in chronic hepatitis B

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Abstract

Rescue antiviral treatment for patients with resistance to preexisting nucleos(t)ide analogues remains a clinical challenge. The correct choice of a first-line treatment of high potency and with a high genetic barrier to achieve sustained long-term suppression of viral replication provides the best chance of preventing treatment failure and the emergence of drug resistance. The management of treatment failure and drug resistance requires a precise and accurate clinical and virologic monitoring. Combination treatment with antiviral drugs that belong to different groups is associated with a lower chance of developing resistance to rescue drugs. To guarantee better control of viral replication in patients with drug resistance, the addition of another drug without a cross resistance profile should be given as early as possible, preferably at the time when genotypic resistance emerges. Long-term surveillance for treatment efficacy and possible emergence of drug resistance should be continued to prevent the emergence of multidrug-resistant strains.

Key words: Chronic hepatitis B; Antiviral resistance; Rescue treatment; Multidrug resistance; Cross resistance

Core tip: Proliferation of hepatitis B virus (HBV) is the key driver of liver injury and disease progression, and thus sustained HBV suppression is of paramount importance in the management of chronic hepatitis B. Long-term antiviral treatment is usually required to achieve sustained suppression of HBV. However, antiviral drug resistance is a serious problem of long-term antiviral treatment, and this poses a critical challenge. Prevention and proper management of antiviral drug resistance are decisive to long-term success of treatment.

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BACKGROUND TO THE DEVELOPMENT OF ANTIVIRAL DRUG RESISTANCE

Although potent antiviral agents for the treatment of chronic hepatitis B (CHB) are currently available, total eradication of hepatitis B virus (HBV) remains practically impossible. Data on the natural history of CHB and the clinical effectiveness of long-term antiviral treatment emphasize the paramount importance of prolonged viral suppression to very low levels. Though, nucleos(t)ide analogues are associated with good viral suppression, as shown by the reduction of serum HBV-DNA levels, viral resistance still is a problem in long-term treatment.

Replication of HBV-DNA occurs *via* a RNA intermediate. In the nucleus of the hepatocyte, host and viral polymerases repair the partially relaxed circular genome of HBV to a fully double stranded covalently closed cir-

Table 1 Classifications and context cross-resistance profiles of antiviral drugs in chronic hepatitis B[8]

Classification	Amino acid substitution in the rt domain	LAM	LdT	ETV	ADV	TDF
	Wild-type	S	S	S	S	S
LAM + LdT resistance	M204I/V	R	R	I	S	S
ADV resistance	N236T	S	S	S	R	I
LAM + LdT + ADV (multi-drugs) resistance	A181T/V	R	R	S	R	I
ADV + TDF resistance	A181T/V + N236T	R	R	S	R	R
ETV resistance	L180M + M204I/V ± I169 ± T184 ± S202 ± M250	R	R	R	S	S
TDF resistance	A194T	R	S	S	NA	R

Adapted and modified from reference [8]. LAM: Lamivudine; LdT: Telbivudine; ETV: Entecavir; ADV: Adefovir; TDF: Tenofovir; S: Sensitive; I: Intermediate; R: Resistant; NA: Not available.

cular DNA (cccDNA). The cccDNA serves as a template for the transcription of all the HBV messenger RNA (mRNA). The viral RNAs include the pregenomic RNA, which serves as both the template for reverse transcription and for the core and polymerase synthesis, as well as the 3 subgenomic mRNAs necessary for the translation of the envelop protein and X protein[1,2].

The error-prone HBV reverse transcription (rt)-polymerase causes a high nucleotide substitution rate, generating a population of viral variants or quasispecies capable of rapidly adapting to endogenous (host immune response) and exogenous selection (antiviral treatment) pressures. The spontaneous mutation rate for HBV is estimated to be 1.4×10^{-5}-3.2×10^{-5} nucleotide substitutions per site and per cycle[3,4]. Concerning the high viral replication rate of more than 10^{11} virions per day[5], at least 10^{10} point mutations could occur in a HBV genome every day. Given the whole genome length of 3.2 kb, all possible single base changes can be produced in a day. The error-prone process of HBV replication, in which errors occur due to the absence of a proof-reading mechanism during the intermediate step of viral replication through reverse transcription, is responsible for the frequent incorporation of inaccurate nucleotides. A major obstacle for the generation of a new viral strain in this background of extremely high mutational frequency is the intrinsic frameshift overlapping organization of the four open reading frames of the HBV genome. However, due to the high mutational rate, it is not unusual to observe drug-resistant HBV already present in a viral population that has not yet been exposed to any nucleos(t)ide analogues.

Effective treatments have been developed for CHB that significantly reduce the morbidity and mortality. Treatment efficacy can be affected by factors such as the development of adverse effects, compliance to the drug, previous treatment with suboptimal regimens, infection with drug resistant viral strains, inadequate exposure due to the pharmacological properties of the particular drug(s) and individual genetic variation. Five drugs belonging to the nucleos(t)ide analogues have been approved for treatment of CHB in most parts of the world[6]. The nucleos(t)ide analogues directly inhibits the reverse transcriptase activity of the HBV polymerase. The approved nucleos(t)ide analogues include lamivudine (LAM), a synthetic deoxy cytidine analogue with an unnatural L-conformation, and related L-nucleoside, telbivudine (LdT; β-L-thymidine). A second group, the acyclic phosphonates include adefovir dipivoxil (ADV), a prodrug for the acyclic 2'-deoxy adenosine monophosphate analogue adefovir, and the structurally similar tenofovir (TDF). A third group contains a D-cyclopentane sugar moiety and has the most potent anti-HBV drug discovered to date, the deoxy guanosine analogue entecavir.[7] This structural subgroups of the nucleos(t)ide analogues is clinically useful because it helps subgrouping the classifications of drug resistance of nucleos(t)ide analogues and guides rescue antiviral treatment according to the cross-resistance profile (Table 1)[8].

Antiviral drug resistance is defined as the decreased susceptibility of a virus to the inhibitory effect of a drug, which results from a series of adaptive mutations under the selection pressure of antiviral treatment. Two types of mutations have been identified: primary resistance mutations, which are directly responsible for the associated drug resistance, and secondary or compensatory mutations, which occur in order for the virus to facilitate replication competence, because the primary resistance mutations may be associated with a reduction in replication fitness[9]. Replication fitness refers to the ability of a virus to replicate under the selective forces. Usually, mutant viruses show less replication fitness; however, over time, secondary mutations, such as rt80, rt180, and rt173 develop after the initial primary rtM204I/V mutation, which restores the functional defects of viral polymerase caused by the primary resistance mutations[10-13].

FACTORS ASSOCIATED WITH THE EMERGENCE OF ANTIVIRAL DRUG RESISTANCE

The likelihood of the emergence of drug resistance depends on the baseline characteristics of the patients, viral factors, drug properties, and treatment regimens. Male gender, older age, high body mass index, high alanine aminotransferase (ALT) level, high HBV-DNA concentration, high histological score (indicating a higher degree of necroinflammation), and the presence of core promoter mutations are reported to be associated with

a higher risk of LAM resistance[14-19]. A few studies have shown that the HBV genotypes A and D are associated with higher rates of LAM-resistant and ADV-resistant mutations, respectively[20-23]. Some correlations between the genotypes of HBV and the selection of specific mutations might exist; however, most studies have shown that HBV genotypes have no relevance to the treatment response and the rate of emergence of drug resistant mutations[24-27].

Another important factor associated with the emergence of drug resistance is the persistence of viral replication during antiviral treatment. Yuen *et al*[28] found that the rate of emergence of LAM-resistant HBV strain was directly proportional to the HBV-DNA concentration at week 24 after treatment (8%, 13%, 32%, and 64% for patients with 24-wk HBV-DNA concentration lower than 200 copies/mL, 3 $\log_{10}$ copies/mL, 4 $\log_{10}$ copies/mL, and 4 $\log_{10}$ copies/mL and higher, respectively, at a median follow-up of 29 mo). Fukai *et al*[29] also found that patients with undetectable HBV-DNA by PCR at week 24 of LAM treatment had a substantially lower rate of virologic breakthrough. Multivariate analysis including the variables of pretreatment ALT level, pretreatment HBV-DNA level, and HBV-DNA level at week 24 showed that the HBV-DNA level at week 24 was the only independent variable associated with the occurrence of a virologic breakthrough. The GLOBE trial, the phase Ⅲ multicenter trial of LdT, also showed the importance of HBV-DNA suppression at week 24 for the emergence of antiviral resistance. Eighteen of 203 (9%) hepatitis B e antigen (HBeAg)-positive patients and 11 of 177 (6%) HBeAg-negative patients with undetectable HBV-DNA at week 24 had LdT resistance at year 2, compared to 46 of 107 (43%) HBeAg-positive patients and 7 of 10 (70%) HBeAg-negative patients with an HBV-DNA concentration of more than 4 $\log_{10}$ copies/mL at week 24[30]. Because of the slower and less potent antiviral activity of ADV, the on-treatment HBV-DNA concentration was assessed at week 48, instead of week 24. Five of 89 (6%) patients with an HBV-DNA concentration of less than 300 copies/mL at week 48 had ADV resistance at 192 wk, compared to 17 of 35 (49%) patients with an HBV-DNA concentration of more than 3 $\log_{10}$ copies/mL[31]. All of the above-mentioned studies stressed the importance of rapid and profound suppression of viral replication to minimize the emergence of drug resistant HBV during long-term treatment with nucleos(t)ide analogues.

MANAGEMENT OF ANTIVIRAL DRUG RESISTANCE

The management of treatment failure has changed significantly in recent years. Actually, treatment failure can be expanded to include a partial virologic response as well as the classic virologic breakthrough with the availability of potent antiviral drugs and precise virologic monitoring tools. Compliance to antiviral drugs in all patients should be closely monitored and reinforced when necessary and antiviral drug resistance should be managed according to the resistance testing profile of the patient's specific HBV polymerase DNA sequence, in the context of the available cross resistance data (Table 1).

LAM-RESISTANT HBV

The treatment strategy for LAM-resistance can also be applicable to LdT-resistance because of the shared drug-resistance profile (rtM204I/V) between LAM and LdT. The rtM204I and rtM204V mutations refer to the substitution of methionine with isoleucine or valine, respectively, at codon 204 of the reverse transcriptase gene. Previously these mutations were called YMDD mutations, but the terminology is no longer recommended[32]. rtM204V mutation emerges during LAM treatment; however, rtM204I can develop during the administration of LAM, LdT, or clevudine[30,33-35]. A rtM204V mutation may commonly be associated with rtL180M, but not with rtM204I mutation[36]. These mutations are sensitive to ADV and TDF, but they exhibit cross-resistance to ETV and show an eight-fold decrease in sensitivity. Kim *et al*[37] have shown that the biochemical response at 12 mo of ADV add-on LAM combination treatment was better in patients with an rtM204I mutation than rtM204V+ rtM204I/V mutations. Additionally, early treatment failure was more common in patients with rtM204V+ rtM204I/V mutations than with an rtM204I mutation. The rtA181T mutation has been detected in 5% of LAM-resistant patients. This mutation exhibits cross resistance to ADV, but remain sensitive to ETV[38].

A pilot study which compared the antiviral efficacy of ADV monotherapy with ADV add-on LAM combination therapy against LAM-resistant HBV infection found a comparable reduction of the viral load (-4.4 $\log_{10}$ copies/mL *vs* -3.59 $\log_{10}$ copies/mL, respectively) and normalization of the serum ALT level (53% *vs* 47%). However, a transient ALT flare was found in 37% of the patients in the ADV monotherapy group[39]. Therefore, switching to ADV monotherapy or short-term (2-3 mo) ADV-LAM combination treatment during rescue ADV treatment to prevent a transient ALT flare was recommended. The rate of ADV resistance in LAM-resistant patients was shown to be as high as 18% at 1 year, compared with 0% in LAM-naïve patients[40]. A study by Yeon *et al*[41] for 67 patients with LAM-resistance who were switched to ADV reported a cumulative ADV resistance rate of 6% and 25% at years 1 and 2, respectively. According to a study from Hong Kong, for 56 patients with LAM-resistance, the cumulative occurrence rate of ADV-resistance at 2 years was 18% for patients who had switched to ADV and 7% for patients who had ADV added to LAM[42]. A recent study of ADV add-on LAM combination treatment for patients with preexisting LAM-resistance showed that the cumulative ADV resistance rates were 1%, 2%, 4%, and 4% for the first 4 years[43]. Therefore, it seems likely that the ADV-resistance rate in patients with preexisting LAM-resistance can be greatly reduced by

ADV add-on LAM rather than switching to ADV.

The timing of the ADV add-on for patients with preexisting LAM-resistance is another crucial factor for better viral suppression. A study performed by Lampertico *et al*[44] showed that the addition of ADV at the time when the HBV-DNA concentration was 3-6 $\log_{10}$ copies/mL and the serum ALT was normal resulted in 100% of the 74 HBeAg-negative patients with preexisting LAM-resistance achieving an undetectable HBV-DNA level at 3 mo. This was compared with only 46% of the patients with an HBV-DNA concentration of more than 6 $\log_{10}$ copies/mL and a high serum ALT level at the time of the addition of the ADV. Thus, the addition of ADV as early as possible (at the time of the detection of genotypic resistance, if possible) is the best strategy for the rescue treatment of patients with LAM resistance.

ETV has also been evaluated as a rescue treatment option for patients with Lam resistance. A study by Kim *et al*[45] showed that ETV 1.0 mg daily for 24 patients with preexisting LAM resistance had a mean $\log_{10}$ HBV-DNA concentration reduction of 2.89, 3.34, and 3.71 at 6, 12, and 24 mo from the baseline. In comparison, ADV add-on LAM combination for 36 patients with preexisting LAM resistance had a mean $\log_{10}$ HBV-DNA concentration reduction of 4.17, 4.63, and 4.86 at 6, 12, and 24 mo from the baseline. This result was statistically analyzed and it was concluded that ADV add-on LAM combination therapy significantly suppressed $\log_{10}$ HBV-DNA to a greater extent than ETV monotherapy at 3, 6, and 12 mo after the initiation of rescue antiviral treatment. Additionally, viral breakthrough and genotypic resistance were detected in six (25.0%) patients receiving ETV monotherapy, whereas no case of genotypic resistance was detected in the ADV add-on LAM combination therapy group 24 mo after the initiation of each antiviral treatment. Although the genotypic resistance rate of ETV is as low as 1.2% at year 5 in treatment-naïve patients, it has been reported that the cumulative rates of ETV genotypic resistance in patients with preexisting LAM-resistance are 6%, 15%, 36%, 46%, and 51% from years 1 to 5[46]. ETV is probably inferior to early ADV add-on for the treatment of LAM-resistant HBV.

TDF has shown potent antiviral activity against LAM-resistant HBV as well as against wild type HBV[47,48]. In a study of 53 patients with LAM-resistance and HBV-DNA of more than 6 $\log_{10}$ copies/mL who received TDF, there was a reduction in the HBV-DNA concentration of more than 5 $\log_{10}$ copies/mL at week 48 compared to 3 $\log_{10}$ copies/mL in those who received ADV[47]. An HBV-DNA concentration of less than 400 copies/mL was achieved in all TDF-treated patients compared with only 44% of patients treated with ADV. In a recent study with a longer follow-up period of 23 mo, TDF monotherapy resulted in 100% HBV-DNA undetectability among LAM-resistant CHB patients[49]. Therefore, treatment strategies which include TDF seems to be more effective than those involving ADV for rescue treatment in patients with LAM-resistance. However, there is a report of TDF resistance in patients with LAM-resistance who received TDF monotherapy, so the efficacy of TDF monotherapy requires further verification[50]. One recent study showed that among 109 LAM-resistant CHB patients, TDF plus LAM combination treatment was more efficacious in reducing the HBV-DNA level than TDF monotherapy, ADV monotherapy, and ADV add-on LAM combination therapy[51]. More recently, combination treatment of TDF plus LdT produced a higher rate of virologic response (defined as a HBV-DNA reduction of more than 2 $\log_{10}$ copies/mL) than combination therapy of TDF plus LAM after 12 mo of treatment[52].

ADV-RESISTANT HBV

Development of resistance to ADV is slower compared to LAM, with the reported rate being 2% at 2 years and 29% at 5 years[31,53]. The primary mutations associated with ADV-resistance are rtN236T and rtI233V in the D domain and rtA181T/V in the B domain[21,31,54-56]. The rtN236T mutant remains sensitive to LAM, LdT, and ETV with less than 3-fold change in the IC_{50} and has a moderately decreased replication capacity compared to wild type HBV[53,57]. The rtA181T/V mutation is associated with decreased susceptibility to LAM, LdT, and ETV (8- to 16-fold) but is sensitive to TDF (about 2-fold change in IC_{50})[58,59]. TDF is effective in suppressing HBV replication in patients who exhibiting LAM-resistance who have failed to respond adequately to ADV, and in patients resistant to both LAM and ADV[60]. However, reduced sensitivity to TDF was demonstrated in ADV-resistant HBV, indicating potential cross-resistance[49]. Therefore, adding emtricitabine (FTC) or LAM to TDF could be a more appropriate treatment strategy than TDF monotherapy in patients with ADV resistance. Actually, the addition of FTC led to a further decrease in the serum HBV-DNA level in patients with ADV resistance and a suboptimal response to TDF monotherapy[61].

ETV has been demonstrated to be effective in suppressing the replication of HBV in patients with ADV-resistance. ETV is effective against both rtA181T/V and rtN236T mutant HBV strains[62-65] because ETV does not possess cross-resistance with ADV[38]. Combination treatment of ADV plus ETV is considered to be a better treatment option because the selection of LAM-resistant strains during ETV-monotherapy can result in subsequent ETV-resistance[66]. Combination treatment of ETV and TDF can also be a treatment option for multidrug resistant HBV infection which includes ADV resistance (especially rtA181T/V)[67].

ETV-RESISTANT HBV

Few clinical studies have investigated the treatment of ETV-resistant HBV. ETV-resistant HBV is still sensitive to ADV, and ADV can be considered to be an initial treatment option in CHB patients with ETV-resistance. Clinical studies indicated that ADV was effective in suppressing the replication of ETV-resistant HBV[68,69]. Com-

Table 2 Recommendations of guidelines for rescue therapy in chronic hepatitis B patients with antiviral drug resistance

Drugs to which antiviral resistance developed	AASLD (2009)[74]	EASL (2012)[6]	APASL (2008)[75]
LAM	Add ADV or TDF Stop LAM, switch to Truvada®[1]	Switch to TDF Add ADV, if TDF is not available	Add-on ADV therapy Switching to ETV therapy (1 mg/d) is an option Switching to interferon-based therapy is an option
LdT	Add ADV or TDF Stop LdT, switch to Truvada®	Switch to TDF Add ADV, if TDF is not available.	Add-on ADV therapy Switching to interferon-based therapy is an option
ADV	Add LAM[2] Stop ADV, switch to Truvada® Switch to or add ETV[2]	If nucleoside-naive before ADV then switch to ETV or TDF If the patient has high viremia then switch to ETV If there is prior LAM resistance then switch to TDF or add a nucleoside analogue	For LAM-naive patients who develop drug resistance while on ADV, add-on or switching to LAM, LdT, or ETV is indicated Switching to interferon-based therapy is an option
ETV	Switch to TDF or Truvada®	Switch to or add TDF Add ADV, if TDF is not available	

[1]Truvada® = combination pill with emtricitabine 200 mg and TDF 300 mg; [2]Durability of viral suppression unknown, especially in patients with prior LAM resistance. AASLD: American Association for the Study of the Liver Diseases; EASL: European Association for the Study of the Liver Diseases; APASL: Asian-Pacific Assocation for the study of the Liver Diseases; LAM: Lamivudine; LdT: Telbivudine; ETV: Entecavir; ADV: Adefovir; TDF: Tenofovir.

bination treatment of ADV plus ETV would be a more appropriate treatment option for reducing ADV resistance and improving antiviral efficacy[66]. TDF is reported to be effective in suppressing the replication of ETV-resistant HBV. In most of the CHB patients with ETV-resistant HBV who showed persistent viremia after LAM plus ADV treatment, HBV-DNA became undetectable after 6 mo treatment of TDF[70].

TDF-RESISTANT HBV

There are no data on the management of TDF resistance. An in vitro study showed that replication of the rtA194T mutant was suppressed effectively by ETV and intermediately by LdT[71].

MULTIDRUG RESISTANCE

Although most HBV strains are resistant to a particular nucleo(t)ide analogue, this resistance can be effectively suppressed by using a nucleo(t)ide analogue from a different structural group. However, multidrug resistance might become a problem in the future. Prolonged and sequential exposure to nucleo(t)ide analogues promotes the formation of clusters of mutations such as rtA181T/I233V/N236T/M250L, all on the one dominant HBV genome, and these clusters are associated with multidrug resistance[72]. To avoid the development of multidrug resistant HBV, efforts should be made to achieve maximal viral suppression with a selection of drugs that have complementary cross-resistance profiles.

SAFETY OF RESCUE ANTIVIRAL TREATMENT

The frequencies of the occurrence of serious adverse events among the ETV 1.0 mg monotherapy, ADV 10 mg monotherapy, and ADV add-on LAM combination treatment were reported to be similar and most of the adverse events were mild-to-moderate in severity[45]. Reported serious adverse events included abdominal, nausea and diarrhea on ETV 1.0 mg monotherapy, and elevation of serum creatinine level in ADV monotherapy and ADV add-on LAM combination treatment. No patients with TDF rescue treatment were reported to develop renal toxicity, defined as a decrease of eGFR more than 20% from baseline. No cases of hypophosphatemia or other adverse events associated with TDF therapy were observed[73].

RECOMMENDATIONS OF GUIDELINES FOR RESCUE THERAPY IN PATIENTS WITH ANTIVIRAL DRUG RESISTANCE

Guidelines can provide evidence-based framework of judgement for determining the most appropriate rescue therapy in CHB patients with antiviral drug resistance; however, individualized and flexible approaches are needed in each patient, considering the patient's preference, physician's experiences, socioeconomic and reimbursement environment of each patient and physician, and progress in knowledge for chronic hepatitis B. Recommendations of guidelines for rescue therapy in CHB patients with antiviral drug resistance are summarized in Table 2.

SUMMARY OF ANTIVIRAL DRUG EFFICACIES IN RESCUE SETTINGS

Virologic and serologic responses to various rescue therapies were summarized in Table 3.

Table 3 Antiviral drug efficacies in rescue settings

Virologic, serologic, and biochemical responses	ADV monotherapy[76]	ETV monotherapy[45,46]	ADV + LAM combination therapy[43]	ADV + LdT combination therapy[77]	ADV + ETV combination therapy[78,79]
Patients with undetectable HBV-DNA (%)					
1 yr	22.8	54.5	61	70.3	88.8
2 yr	48.9	50.0	70		97.8
3 yr	56.8		79		
4 yr	60.3		82		
5 yr	60.3				
Cumulative probability of genotypic resistance (%)					
1 yr	4.4	6	0.7	0	0
2 yr	18.4	15	0.9		0
3 yr	34.3	36	1.3		
4 yr	52.3	46			
5 yr	65.6	51			
Cumulative probability of HBeAg seroconversion (%)					
1 yr	7.3	0		9.67	15.6
2 yr	12.7	0			26.7
3 yr	15.0		24		
4 yr	17.0				
5 yr	17.0				
Cumulative probability of ALT normalization (%)					
1 yr	80.3	77.3	84	64	100
2 yr	83.2	80	87		100
3 yr	86.7		89		
4 yr	88.2				
5 yr	88.2				

ADV: Adefovir; ETV: Entecavir; LAM: Lamivudine; LdT: Telbivudine; TDF: Tenofovir; HBV-DNA: Hepatitis B virus deoxynucleic acid; HBeAg: Hepatitis B e antigen; ALT: Alanine aminotransferase.

CONCLUSION

To prevent and minimize the emergence of drug resistance, nucleo(t)ide analogues that cause rapid viral suppression with a high genetic barrier to resistance should be the treatment of choice. Clinical studies have shown that drugs with a high genetic barrier to resistance, such as ETV and TDF, have significantly lower rates of resistance compared to those with a low genetic barrier to resistance, such as LAM, ADV and LdT. The first choice of an antiviral drug should include a highly potent agent with a high genetic barrier in order to achieve sustained long-term suppression of viral replication, thereby providing the best chance of achieving the primary goal of treatment - the prevention of liver disease progression. Management of treatment failure due to the emergence of antiviral resistance requires precise clinical and virologic monitoring and rescue treatment with the appropriate complementary drug(s), with checking of their cross-resistance profile as early as possible. To achieve a better clinical response in CHB patients with antiviral drug resistance, the addition of another nucleo(t)ide analogue from a different structural group without cross-resistance should be given, preferably at the time when genotypic resistance emerges. Although antiviral drug resistance remains a major clinical concern, continuous virologic monitoring with sensitive and quantitative tools and the development of a new generation of antiviral agents with a better potency and high genetic barrier to resistance have brought major improvements in the management of patients with CHB.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (9): Hepatitis B virus

Phage display creates innovative applications to combat hepatitis B virus

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Core tip: Hepatitis B virus (HBV) poses a major health problem worldwide and currently there is no effective treatment for HBV infection. Treatments of patients with nucleoside analogues have resulted in the selection of vaccine escape and drug resistant mutants. Most frightening, HBV is now linking arms with human immunodeficiency virus to threaten the world. Phage display has been employed extensively to solve these life-threatening problems. This article reviews critically the innovative applications of phage display in epitope mapping and the development of vaccines, therapeutic agents, diagnostic reagents, as well as gene and drug delivery systems.

Tan WS, Ho KL. Phage display creates innovative applications to combat hepatitis B virus. *World J Gastroenterol* 2014; 20(33): 11650-11670 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i33/11650.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i33.11650

Abstract

Hepatitis B virus (HBV) has killed countless lives in human history. The invention of HBV vaccines in the 20th century has reduced significantly the rate of the viral infection. However, currently there is no effective treatment for chronic HBV carriers. Newly emerging vaccine escape mutants and drug resistant strains have complicated the viral eradication program. The entire world is now facing a new threat of HBV and human immunodeficiency virus co-infection. Could phage display provide solutions to these life-threatening problems? This article reviews critically and comprehensively the innovative and potential applications of phage display in the development of vaccines, therapeutic agents, diagnostic reagents, as well as gene and drug delivery systems to combat HBV. The application of phage display in epitope mapping of HBV antigens is also discussed in detail. Although this review mainly focuses on HBV, the innovative applications of phage display could also be extended to other infectious diseases.

INTRODUCTION

Hepatitis B virus (HBV), which causes liver cirrhosis and hepatocellular carcinoma (HCC), is one of the greatest killers in human history. It is 200 times more infectious than human immunodeficiency virus (HIV) and so far has killed more people than HIV. HBV poses a serious global health problem, about one third of the world's population have been infected by this virus, of which 370 million are chronic carriers and about one million people die each year[1]. The virus is commonly present in human population, at least 7% of the people in South East Asia, China and Africa are chronically infected by HBV.

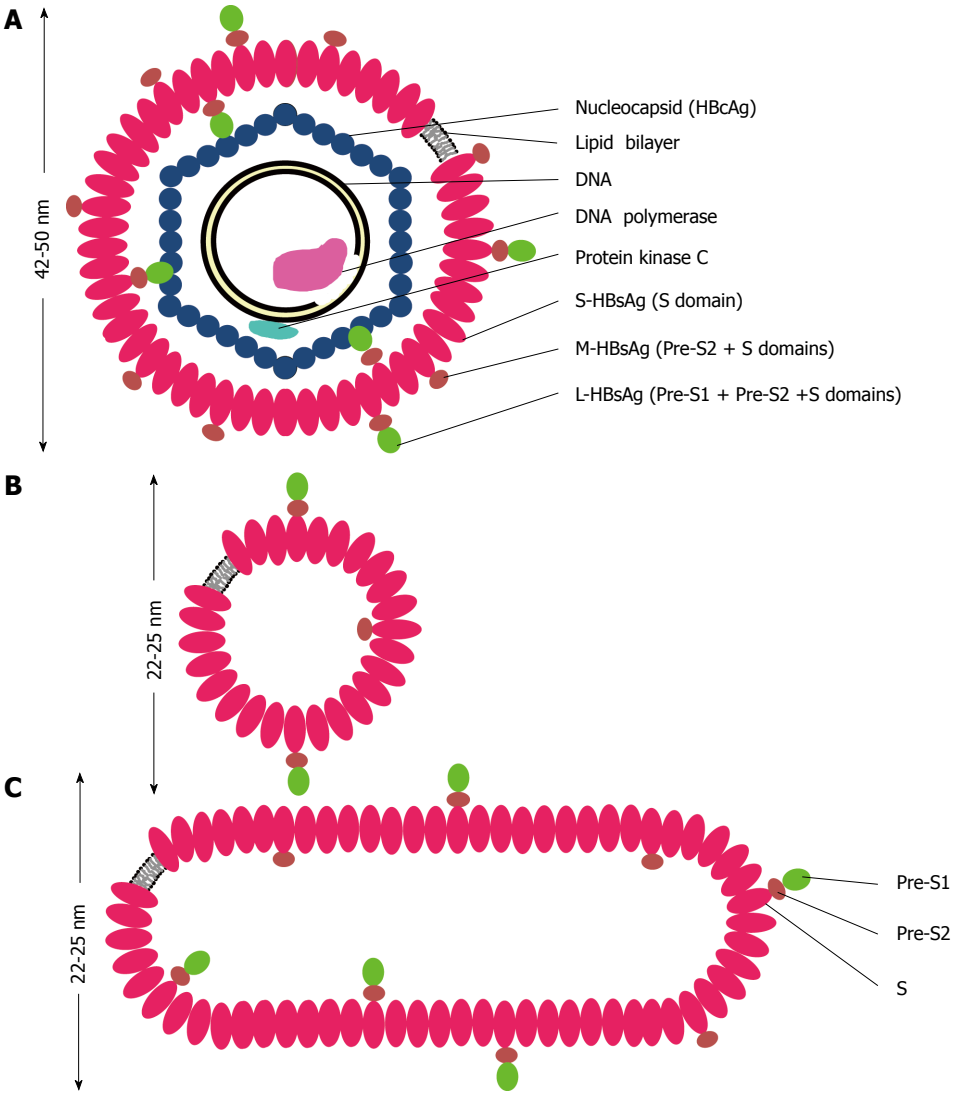

Figure 1 Schematic representations of hepatitis B virus virion (A), spherical (B) and filamentous (C) particles. The envelope of hepatitis B virus (HBV) virion or the Dane particle (A) contains three forms of hepatitis B surface antigen (HBsAg): large (L-) HBsAg has the PreS1, PreS2 and S domains; middle (M-) HBsAg contains the PreS2 and S domains; and small (S-) HBsAg has only the S domain. The representations of the L-, M-, and S-HBsAg have no quantitative or positional significance. The L-HBsAg interacts with the viral capsid which is made of many copies of the core protein (HBcAg). The capsid encapsidates a partially double stranded DNA molecule, a DNA polymerase containing the primase and reverse transcriptase activities. The protein kinase C phosphorylates the capsid protein. The diameter of HBV virion is about 42 nm when it is stained negatively and observed under a transmission electron microscope, but it appears bigger in cryo-electron microscopy. The spherical (B) and filamentous (C) particles have a diameter about 22 nm in negative staining and appear bigger in cryo-electron microscopy. The length of the filamentous particle varies. Both the non-infectious particles contain the L-, M-, S-HBsAg and lipid.

The virus is now classified in the family of *Hepadnaviridae* and it has a narrow host range, infecting only human and other higher primates such as chimpanzees and orangutans. The modes of HBV transmission include blood transfusion, unprotected sexual contact, contaminated needles and syringes, and *via* perinatal transmission from an infected mother to her baby during childbirth.

Hepatitis B surface antigen (HBsAg) was accidentally discovered in 1960's by an American doctor, Baruch Blumberg, in the blood of an Australian aborigine, when he was studying the inherited variations in human beings[2]. In 1970, for the first time, Dane *et al*[3] reported the observation of the virus particles under a transmission electron microscope. The infectious particle or known as the Dane particle, is roughly spherical with a diameter about 42 nm (Figure 1). This particle can be found in the blood of a chronically infected patient and its three-dimensional structure has been determined by cryo-electron microscopy[4]. The virion is enveloped by a lipid bilayer derived from the host cell membranes[5]. Associated with the lipid are three distinct but related forms of surface protein (HBsAg): S- (small), M- (middle) and L- (large) HBsAg.

Inside the envelope is the viral nucleocapsid which is made of many copies of core protein or commonly known as core antigen (HBcAg). Within the capsid is a partially double-stranded DNA genome. The polymerase protein (P) which has reverse transcriptase and DNA-dependent DNA polymerase activities is covalently linked to a partially double-stranded circular DNA genome of

about 3.2 kb.

Apart from the virion, another two distinct forms of non-infectious particles are also observed in the serum of a chronic carrier. They appear as spheres or filaments with a diameter of about 22 nm when observed under an electron microscope (Figure 1). The filamentous particles have different length. The amount of these non-infectious particles is about 1000- to 100000-fold in excess compared to the virion[6] and it is thought to serve as decoys to fool human's immune system.

On the other hand, not all viruses are harmful to human beings. Viruses that infect and replicate in bacteria or known as bacteriophages (phages) are believed to control bacterial populations on this planet. They are distributed in oceans, rivers, soils, animals, insects and places populated by bacteria. These useful viruses were discovered separately by Frederick Twort and Félix d'Hérelle in 1910's[7], about 50 years earlier before HBV was discovered. They occur in a wide variety of different sizes and shapes with DNA or RNA as their genomes. One may not be aware of phages' impacts on the global economy and the advancement of science and technology. In fact, they were used widely as anti-bacterial agents in the early and mid-20th century before antibiotics were discovered. Phages also contributed significantly in understanding the fundamentals of DNA replication, transcription, translation, recombination and regulation. Some modification enzymes used in molecular biology, including ligase and RNA polymerase are isolated from these viruses. Phages, such as M13 and lambda, are employed as DNA cloning vectors for protein productions and determination of nucleotide sequences in organisms, including the human being[8].

On June 14, 1985, the Science, published an article by George P. Smith, describing an innovative method to display peptides on a filamentous phage by fusing their coding sequences to the phage's genome[9]. This article marks the beginning of phage display, so simple that it links a genotype and a phenotype physically in a single phage particle, and so powerful that it allows the amplification of these two elements in billion folds in bacteria. Immediately, phage display ignited a spark of interest among scientists around the world, who incorporated, refined and applied it to almost every field of biological sciences, which include drug discovery, antibody engineering, epitope mapping, vaccine development, gene and drug delivery, development of diagnostic reagents, organ targeting, enzyme technology, protein-protein interactions and protein-DNA interactions. At the time of writing this manuscript, there are more than 20000 scientific articles about phage display on Google Scholar, demonstrating the impact of this method on current global economy, science and technology. Phage display has now developed into one of the most important tools in biological research, and of course it has also been employed extensively to study HBV particularly its innovative applications to control this virus. However, to the best of our knowledge, so far there is no review paper summarizing the applications of phage display on HBV research. Therefore, the main aim of this paper is to provide a comprehensive and critical review on the innovative applications of phage display to combat HBV. In order to achieve this aim, this article is divided into five sections based upon the main strategies that have been used to control and manage HBV.

VACCINE DEVELOPMENT

Edward Jenner's brilliant idea about small pox vaccination in 18th century, also worked effectively for HBV vaccination in 20th and 21st centuries to prevent the spread of HBV, and of course this has saved countless lives worldwide. The first HBV vaccine was prepared by Baruch Blumberg and Irving Millman by using the non-infectious HBV particles (Figure 1) purified from infected sera[10]. This invention was patented by Fox Chase Cancer Center, licensed to Merck and Company, Inc. and approved by the U.S. Food and Drug Administration (FDA)[10]. In 1981, Merck marketed the first hepatitis B vaccine, Heptavax, but was discontinued in 1990 and replaced by a recombinant DNA vaccine[11].

The invention of recombinant DNA technology in 1970s allows the genetic materials from different organisms to be joined together, artificially introduced into an organism and expressed in the host. This innovative technology was quickly employed by Kenneth Murray, from the University of Edinburgh and also a co-founder of Biogen Inc., to insert the HBV DNA in a bacterial plasmid and to produce the recombinant proteins in *Escherichia coli* (*E. coli*)[12,13]. The experiment was successful and the first patent applications were filed in December 1978[14]. In early 1980s, the HBsAg was successfully produced in yeast by using recombinant DNA technology[15-17] and was demonstrated to protect chimpanzees from HBV infection[18,19]. This second generation vaccine is safer compared to the non-infectious particles purified from the sera of infected patients as the latter may be contaminated with other human pathogens. Production of this recombinant vaccine, when compared with the plasma-derived vaccine, is less laborious and less time-consuming. From the commercial aspect, Biogen Inc. licensed the patent to SmithKline Beecham, and almost at the same time Chiron Corporation, founded by William Rutter[20], worked with Merck to commercialize the vaccine. The recombinant vaccine manufactured by Merck (Recombivax HB®) and SmithKline Beecham (Engerix-B®) was eventually approved by the United States FDA in late 1980s[21]. The mass immunization of infants worldwide as recommended by the WHO has dramatically reduced the rate of HBsAg carrier in many countries[22]. For instance, in China the prevalence of HBsAg carriers dropped from 14.6% to 1.4%[23]. In addition, the incidence of HCC also declined by 4-folds in children after the implementation of mass immunization in Taiwan[24], indicating that HBV vaccine could prevent liver cancer.

Currently, there are effective vaccines in the market to prevent HBV infection. Is there a need to invent and

Table 1 Phage display in vaccine discovery

Strategy	Phage (carrier)	Vector (target)	Epitope/mimotope/gene	Vaccination	Results	Ref.
Display of immunogens on phage particles	T7 (10B)	T7 Select 415-B	"*a*" determinant of HBsAg (residues 111-156)	Rabbit (subcutaneous) Freund's complete adjuvant	Anti-HBsAg antibody induced	Tan *et al*[78]
	M13 (pⅧ)	pC89 with helper phage VASM13	$HBsAg_{28-39}$	Mice (intradermal and intraperitoneal) no adjuvant added	MHC class I restricted HBsAg specific CTL response	Wan *et al*[40]
	M13 (pⅢ)	pCANTAB5E with helper phage M13KO7	PreS1	NA	NA	Kok *et al*[41]
	M13 (pⅢ)	pCANTAB5E with helper phage M13KO7	HBcAg	Mice (intraperitoneal) incomplete Freund's adjuvant	Anti-HBcAg antibody induced	Bahadir *et al*[42]
Identification of epitope or mimotope mimics	M13 nonapeptide library (pⅧ)	(Selected from HBV-infected sera)	Φ13 CRTCAHPGEHA Φ17 CIPFYLSAPQC Φ30 CGPFFLSPTSC Φ41 CGPFFLAASVC	Mice (intraperitoneal) no adjuvant added	Anti-HBsAg antibody induced	Folgori *et al*[54]
	M13 (pⅧ and pⅢ)	(Selected from HBV-infected sera)	Φ13, Φ35 (CVTCDTPPTY) ΦⅢ/17, ΦⅢ41 and ΦpⅢ/13	Mice (intranasal/oral) cholera toxin as adjuvant	Anti-HBsAg antibody induced	Delmastro *et al*[56]
Phage-delivered DNA vaccine	λ	λ-gt11	*HBsAg* gene	Mice (subcutaneous)	Anti-HBsAg antibody induced	Clark *et al*[59]
	λ	λ-gt11	*HBsAg* gene	Rabbits (Intramuscular)	Anti-HBsAg antibody induced	Clark *et al*[61]

NA: Not applicable; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; MHC: Major histocompatibility complex; HBcAg: Hepatitis B core antigen; CTL: Cytotoxic T lymphocyte.

formulate new vaccines? Yes, or at least until the virus is totally eradicated from this planet. Immune-escape mutants with amino acid substitutions, deletions and insertions across the immunodominant region or the "*a*" determinant of HBsAg have been reported widely[25-28]. Besides, a prolonged treatment of chronic hepatitis B patients with nucleotide or nucleoside analogs have resulted in the selection of vaccine escape mutants harboring nucleotide substitutions in their *polymerase* (*pol*) and *S* genes[29]. Most frightening, millions of people worldwide, particularly sub-Saharan Africa and East Asia, are co-infected by HBV and human immunodeficiency virus (HIV)[30]. These people have a higher rate of liver-related mortality compared to those only infected by HIV-1 or HBV alone[31]. In addition, only 20%-70% of HIV-infected patients developed an anti-HBsAg response after a standard HBV vaccination compared to 90%-95% in healthy adult individuals[32]. All the above scientific and medical evidences as well as the viral genetic diversity, justify strongly a continuing need for the development of new HBV vaccines. We believe that phage display can provide alternative solutions to these life-threatening problems. In general, up till now, phage display has been employed extensively to address these problems and can be grouped into three categories based on its applications: (1) display of immunogens on phage particles; (2) identification of epitope or mimotope mimics from sera; (3) phage-delivered DNA vaccine. These applications are summarized in Table 1 and discussed separately in the following sections.

Display of immunogens on phage particles

Several polypeptide carriers have been developed to display or serve as a fusion partner for HBV immunogenic regions. These include HBV capsid (reviewed by[33,34]), ice nucleation protein (INP) located on the outer membrane of *Pseudomonas syringae*[35], heat shock protein 70 from *Mycobacterium tuberculosis* (TBhsp70)[36] and heat shock protein 65[37]. Apart from these potential carriers, phage particles provide an alternative means to display HBV epitopes and mimotopes.

Tan *et al*[38] demonstrated that the T7 phage is an efficient carrier for the highly conformational immunodominant region or denoted as "*a*" determinant of HBsAg (residues 111-156). This region was fused to the C-terminal end of the 10B capsid protein of T7 phage and about 10^{16} copies of reasonably pure immunodominant region can be produced and purified from 1 L culture within 6 h. The recombinant phage, namely T7-$HBsAg_{111-156}$, was demonstrated to be highly immunogenic in rabbits and the immune response was as good as that of human-derived HBsAg, illustrating the potential of the whole recombinant phage particle as a vaccine candidate. The biological and physical features of T7 phage make it an excellent choice to display polypeptides: (1) the robust structure of the phage particle with an icosahedral head allows it to survive in extreme conditions; (2) the icosahedral head is composed of 415 copies of 10B proteins, enabling a high copy number of polypeptides, up to 50 residues, to be displayed on the surface of the phage particle[39]; and (3) T7 phage propagated faster than filamen-

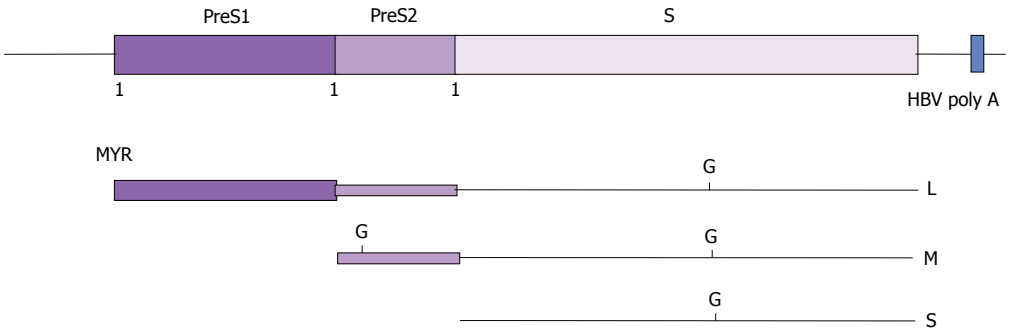

Figure 2 Envelope proteins of hepatitis B virus. The translation products of the *HBsAg* gene are shown as lines of different thickness. Small (S-), middle (M-) and large (L-) HBsAg are translated from a common open reading frame of the *HBsAg* gene by the use of three in-frame initiation codons (1) at the N-termini of PreS1, PreS2 and S. G: Glycosylation; MYR: Myristic acid; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen.

tous bacteriophages and its progenies assembled in the cytoplasm of *E. coli* cells and are released by breaking the host cells, thus the displayed peptides do not have to possess the capability to secrete through the periplasm and the cell membrane, as required by filamentous phages.

Several HBV immunogenic epitopes have also been displayed on filamentous phages as reported by Wan *et al*[40], Kok *et al*[41] and Bahadir *et al*[42]. In the first article, Wan *et al*[40], fused a 12-mer epitope, HBsAg$_{28-39}$, to the pⅧ coat protein of M13 phage. The vector, phagemid pC89, was used in the cloning of the coding sequence and the hybrid phage particles were produced by co-infecting with a helper phage, VASM13. The recombinant phage particles were inoculated into BALB/c (H-2d) mice in the absence of an adjuvant. Interestingly an MHC class I restricted HBsAg specific cytotoxic T lymphocytes (CTL) response was observed after 8 days of inoculation, demonstrating the potential of the recombinant M13 phage particles as potent immunogens without an adjuvant. The pⅧ protein allows a very high copy number of display because an M13 phage particle is made up of about 2700 copies of this major coat protein. On the other hand, the pⅢ protein can only display up to 5 copies of foreign peptides at the tip of a filamentous phage. Kok *et al*[41] displayed the PreS region (163 residues) on M13 phage by using the pCANTAB5E vector with the help of the M13KO7 helper phage. The expression level of the fusion protein, PreS-g3P, was very low, most likely due to the low copy number of the pⅢ protein. Nevertheless, the fusion protein was shown to be antigenic. Recently, Bahadir *et al*[42] fused a larger protein, a full-length HBcAg (about 180 residues), to the pⅢ protein of M13 phage, by using the vector and helper phage as described by Kok *et al*[41]. The recombinant phage particles were shown to be highly immunogenic in BALB/c mice.

The filamentous phage is an excellent immunogenic carrier because: (1) its DNA can be manipulated easily and the viral particle can be conjugated chemically; (2) it does not lyse its host cells and these two major components can be separated easily by a simple centrifugation or any separation methods; (3) it elicits strong antibody responses at low doses and in the absence of an adjuvant[43-45]; (4) its immunogenicity is also enhanced by host lipopolysaccharide (LPS) associated with the purified phage preparation[46,47]; and (5) it elicits T cell responses[48]. The high immunogenicity of the phage particle itself may mask the immunogenicity of the displayed peptides. In order to reduce the phage's immunogenicity, van Houten *et al*[49] deleted the immunodominant region of the pⅢ protein which enhanced and focused the antibody response against the chemically-conjugated synthetic peptide. This approach plus other mutagenesis techniques, amino acid substitution and insertion, can be employed to reduce the immunogenicity of phage particles, be it λ, MS2, P4, T4 or T7.

The use of T4 phage to display HBV epitopes has yet to be reported. Interestingly, the ability of this phage to co-display two immunogenic components on a single phage particle by exploiting two different capsid proteins, Hoc and Soc[50], would be an advantage to display HBV and HIV epitopes together, to prevent the co-infection by these two life-threatening viruses. The limitation of phage display is that the displayed peptide is not post-translationally modified as in eukaryotic systems, but this can be overcome by identification of epitope or mimotope mimics from sera as described below.

Identification of epitope or mimotope mimics

HBsAg are glycoproteins; the S-HBsAg is either glycosylated or un-glycosylated at Asn-146 of the S region (Figure 2) and the M-HBsAg has an additional glycosylation site at Asn-4 of the PreS2 region[51]. A myristyl group is linked to the glycine residue at the N-terminus of L-HBsAg[51], which is required for the virus infectivity[52]. The phosphorylation of HBcAg, the sole monomer of the viral capsid, is believed to facilitate the transport of the viral genome into host's nucleus[53]. All these post-translational processes plus the formation of cysteine bonds and others have yet to be discovered processes, may play significant roles in stimulating both humoral and cellular immune responses. This poses a major challenge to the current recombinant DNA technology and chemical synthesis technology. Our understanding about the diversity and complexity of the cellular and humoral responses of HBV infection is still limited. For a start, the complexity of a random peptide library displayed on phages (about

1 billion different peptide sequences for a 6-mer peptide library), provides a practical approach to dissect the immune response of HBV infection. Both monoclonal and polyclonal antibodies from human sera can be used as substrates for the selection of epitopes or mimotopes that mimic antigen-antibody contact sites (molecular mimicry). For instance, Folgori *et al*[54] successfully identified mimotopes of HBsAg using HBV-infected sera. Some of the selected peptides share similarity with the amino acids located at positions 121-127 of HBsAg. Subsequently, immunization of mice and rabbits with the selected phages harboring the mimotopes mounted cellular[54] and humoral[55] responses resembling those induced by HBsAg. This discovery provides a method to map an immune response by using phage displayed peptide library, without any information about the infected agent. Delmastro *et al*[56] further demonstrated that the mimotope mimics induced specific antibodies when administered orally to BALB/c mice, suggesting the potential of phage display in oral vaccine development.

Phage-delivered DNA vaccine

A direct injection of a plasmid DNA encoding the HBsAg into mouse muscles has led to the production of the viral surface antigens in the tissues which eventually induced both humoral and cell-mediated immune responses[57]. This finding provides an alternative means for HBV vaccine development, apart from the well established virus-based and protein-based vaccines. The application of this DNA-based vaccine was further extended by Mancini *et al*[58], who demonstrated that the HBsAg transgenic mice representing HBV chronic carriers, mounted an immune response after a single intramuscular injection which resulted in a complete clearance of circulating HBsAg. This result opens new inroads in designing more effective ways in treating HBV chronic carriers. A clinical trial of the DNA vaccine in hepatitis-naive human volunteers using a gene gun (PowderJect™ system) as a delivery means into skin cells showed that the vaccine induced protective antibody titers as well as humoral and cell-mediated immune responses. However, up till now, there is no DNA vaccine available in the market to prevent HBV infection or treatment of chronic carriers. Perhaps, a longer time is needed, to overcome all the shortcomings of a DNA vaccine, before it can be used practically for mass inoculation in human. It is hope that phage display could play some roles in leading us towards this ultimate aim.

A novel concept to deliver DNA vaccines by using phage particles was introduced by Clark and March[59]. The *HBsAg* gene was inserted into a phage λ vector containing a DNA vaccine expression cassette and the cytomegalovirus promoter (PCMV). The whole phage particles harboring the recombinant DNA, rather than the naked DNA, were used to immunize mice and rabbits. Interestingly, the anti-HBsAg antibody levels were found to be higher than the standard plasmid-based DNA immunization in these animals[59,60]. Recently, the researchers have compared the phage-delivered HBsAg DNA vaccine with commercially available protein-based vaccine, Engerix B (GlaxoSmithKline Inc.). Amazingly, the results showed that phage-mediated DNA vaccination gave rise to anti-HBsAg antibody levels that were higher than the commercially available recombinant protein vaccine in rabbits immunized intramuscularly[61].

The advantages of phage DNA vaccines include: (1) the DNA is encapsidated by phage coat proteins, thus it is protected from degradation by nucleases; (2) phages are stable at room temperature, which are convenient for storage and transportation; (3) phages grow rapidly in bacterial hosts, hence production and purification of phages are relatively faster, simpler, and cheaper compared to protein- and virus-based vaccines; (4) they can be delivered orally as demonstrated by Delmastro *et al*[56]; (5) the genomes of certain bacteriophages including phage λ can carry a large foreign DNA fragment (up to 15 kb), which allow several genes from different etiological agents to be packaged in single phage particle; and (6) potential vaccine candidates for newly emerging diseases and mutants, particularly those of veterinary importance, could be developed easily and rapidly. Nevertheless, in spite of these advantages and promising results of phage-delivered DNA vaccine in small animals, it is of utmost importance to investigate the distribution, cellular uptake and expression of the DNA at cellular lever as well as their subsequent mechanisms in stimulating cellular and humoral responses. A potential HBV DNA vaccine should also be tested and validated in primates closely related to human, followed by thorough clinical trials.

DEVELOPMENT OF DIAGNOSTIC REAGENTS AND ASSAYS

The life-threatening complications posed by HBV and its global economic impact have always been the driving force for the development of streamlined diagnostic assays. From past to present, and perhaps in the future, the assays for detecting HBV infection focus on the viral antigens, nucleic acids and the antibodies produced by the human body. The appearance of these serological and genetic markers during acute, chronic and occult infections is summarized in Figure 3. The serological markers used routinely in clinical practice are HBsAg, anti-HBsAg, HBeAg, anti-HBeAg and anti-HBcAg (including total anti-HBcAg antibodies and anti-HBcAg IgM). In an acute HBV infection, the HBsAg is the first detectable viral antigen to appear during infection, followed by HBeAg. About three months after the viral infection, the anti-HBcAg is the first antibody to appear, followed by anti-HBeAg and anti-HBsAg[14]. The primary markers for the identification of acute HBV infection are HBsAg and anti-HBcAg. For some lucky people (about 90%-95%), the infection is utterly controlled and resolved by their immune systems. This is observed by the disappearance of the HBsAg, but the anti-HBcAg and anti-HBsAg remain positive which protect the body from further infection. If the infection is not resolved completely, these

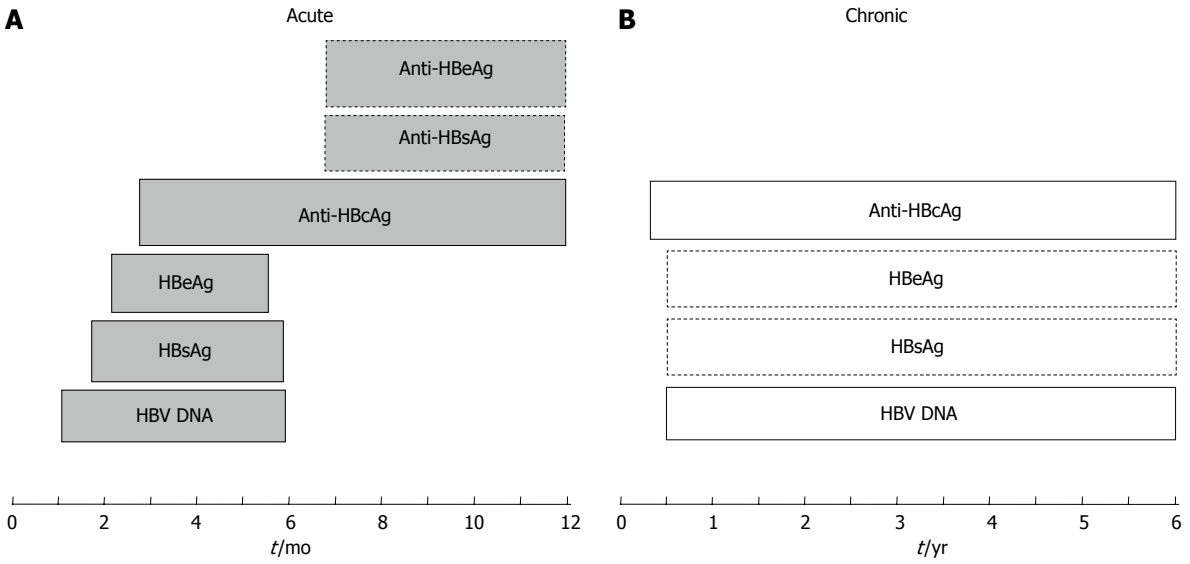

Figure 3 Hepatitis B virus markers in sera during acute (A) and chronic (B) infections. The periods in months (acute infection) and years (chronic infection) are indicated below the boxes containing the markers. The presence of the markers indicated in boxes with broken lines varies in individuals. HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen; HBcAg: Hepatitis B core antigen.

individuals become chronic carriers with the presence of HBsAg and anti-HBcAg. A repeat HBsAg test after six months will determine whether the infection is resolved or chronic. For chronic carriers, HBeAg and anti-HBeAg are tested to measure the level of viral infectivity and seroconversion status[62]. Anti-HBcAg (total) normally indicates a prior infection and it also provides evidence of an occult HBV infection, in which the viral DNA is present without detectable HBsAg[63]. Apart from detecting occult infection, the amount of viral DNA is commonly used to monitor HBV replication, disease progression and also to investigate the responses to drug treatment[62].

A highly sensitive and specific radioimmunoassay (RIA) involving antigen-antibody interaction on a solid phase and radioisotopes was developed in early 1970s for the detection of HBsAg and anti-HBsAg[64-66]. Due to the drawbacks of RIA which involves radioisotopes, special precautions, licensing and equipment are required, therefore it was quickly replaced by ELISA for detecting HBsAg[67], HBeAg[68,69], anti-HBcAg IgM[70], HBcAg[71] and anti-HBeAg[72]. Today, the quantitative real-time PCR is able to monitor the replication of HBV DNA and quantify its amount in different stages of the viral infection[73]. In the advancement of these methods, phage display has demonstrated its potential in generating antibodies and diagnostic reagents. There is no doubt in the importance of phage display in the development of diagnostic assays in the 21st century to combat HBV.

Phage displayed antibodies as diagnostic reagents

Polyclonal antibodies produced in animals have been used widely for the detection of HBV antigens before the introduction of hybridoma technology for the production of monoclonal antibodies. This revolutionary technology involves the fusion of antibody producing spleen cells from mice with immortal myeloma cell lines[74]. The use of monoclonal antibody that interacts with a single epitope on an antigen has increased the specificity of a diagnostic assay and thus reduced cross-reactivity with other antigens. However, the production of monoclonal antibody by using hybridoma technology is time consuming and laborious. In addition, the application of mouse monoclonal antibodies as therapeutic agents is limited by the fact that they are highly immunogenic and cleared rapidly by the human immune system. All these limitations can now be overcome by the rapid advancement of recombinant DNA technology, *in vitro* isolation of antibodies from a combinatorial library and large scale production of antibodies in bacteria. Phage display fulfills these criteria and it is commonly used to display single chain fragment variable (scFv[75]), fragment antigen binding (Fab[76]), the variable heavy domain of camels (VHH) and humans (dAb[77]). These antibody fragments which are smaller than the immunoglobulin (IgG) are illustrated in Figure 4.

A phage-displayed scFv that interacts specifically with HBcAg was constructed by Tan *et al*[78] and the whole phage particle was used to establish a phage-ELISA for detecting HBcAg in human sera. The HBcAg is one of the markers of HBV infection because it is correlated with the infectious particles and also proportional to the level of HBV genome in a serum[71,79]. For the construction of a scFv library, mice were first inoculated with purified HBcAg and total RNA molecules were extracted from their spleens. The genes encoding heavy (V_H) and light (V_L) chains were amplified by PCR and linked by a linker to generate scFv coding regions. These coding regions were then inserted into the pComb3x vector and introduced into *E. coli*. The resultant phage displayed scFv library was panned against HBcAg immobilized on a solid phase and a phage clone that interacted tightly with the immunodominant region of HBcAg was iso-

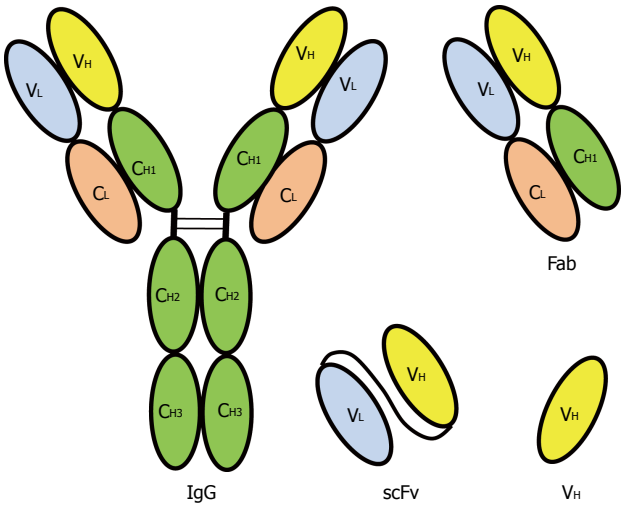

Figure 4 Recombinant antibodies displayed on bacteriophage compared with an immunoglobulin G molecule. Chain structure of a human immunoglobulin G (IgG) molecule. V: Variable region; C: Constant region; H: Heavy chain; L: Light chain; V_H: Variable region of heavy chain; Fab: Fragment antigen binding; scFv: Single chain fragment variable, the V_L and V_H are linked by a peptide linker.

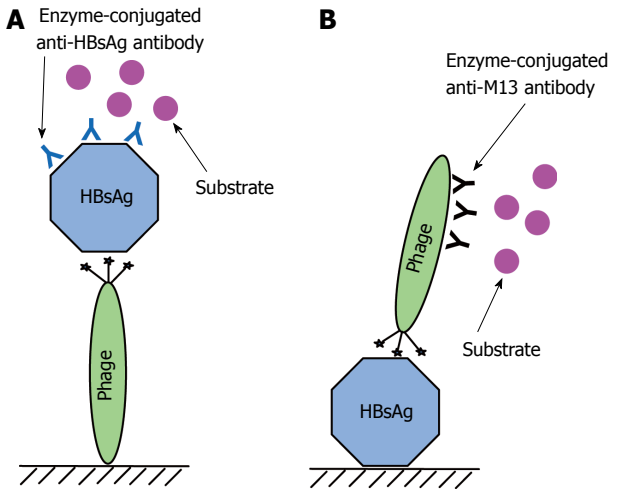

Figure 5 Phage-ELISA for detecting hepatitis B virus surface antigen. A: Major steps used in an assay for detecting hepatitis B virus surface antigen (HBsAg) by immobilizing phage carrying the peptide on a solid support. The phage harboring the peptide is immobilized on a solid support. The unsaturated area of the solid phase is blocked by 10% milk diluent. The sample containing HBsAg is added to interact with the peptide. Mouse anti-HBsAg antibody is added to interact with the captured HBsAg. Then anti-mouse antibody conjugated to an enzyme is added to interact with the anti-HBsAg antibody. Finally, substrates for the enzyme are added to produce a measurable signal; B: Major steps used in an assay for detecting HBsAg immobilized on a solid support using a phage carrying the peptide. A sample containing HBsAg is immobilized on a solid support. The unsaturated area of the solid phase is blocked by 10% milk diluent. The phage harboring the peptide is added to interact with immobilized HBsAg. Anti-M13 phage antibody conjugated to an enzyme is added to interact with the bound phage and substrates for the enzyme are added to produce a measurable signal. ELISA: Enzyme-Linked Immunosorbent Assay.

lated. The selected phage was used to establish a phage-enzyme-linked immuno sorbent assay (ELISA) for detecting HBcAg in human serum samples. This assay was able to detect as low as 10 ng of HBcAg and it did not cross react with HBsAg and HBeAg[78].

A phage displayed Fab that interacts tightly with the PreS1 (residues 37-45) of HBsAg was developed by Kim *et al*[80]. The library was generated by immunizing mice with a PreS1 epitope fused to the N-terminal domain of human thrombopoietin. Phagemid vector pC3-na was used in the construction of the cDNA library. A phage clone harboring the Fab was selected from a biopanning process and demonstrated to bind tightly to the PreS1 region with a K_D of 1.2 nmol/L. The recombinant phage was also shown to interact with HBV particle by an immunoprecipitation assay.

Up till now, the phage displayed antibodies against HBeAg and HBxAg have yet to be reported. In principle, the methods described above can be employed to generate specific recombinant phage antibodies against these two important antigens of HBV. The phage-based diagnostic assays that can be developed are not limited to ELISA, the phage antibodies can also be incorporated into other diagnostic technology such as lateral flow strip assays, proximity ligation assay and flow cytometry. Production of monoclonal antibodies and their related fragments *via* phage display technology are without doubt, simpler, cheaper and faster compared with hybridoma technology.

Phage displayed peptides as diagnostic reagents

The key feature of a diagnostic assay is molecular interactions. In most cases, particularly ELISA, the main interaction involved is between antibody and antigen. To be more precise, the paratope of an antibody interacts with an epitope or a mimotope (discontinuous epitope) on the surface of an antigen. The contact region in an antibody-antigen interaction could be less than 10 residues, therefore, in principle the antibody can be replaced by a peptide which can be selected from a random phage displayed peptide library. This principle was proven correct by Tan *et al*[81] who successfully isolated a phage-displayed cyclic peptide bearing the sequence ETGAKPH that interacts tightly with the immunodominant region of HBsAg. The paratope mimic, ETGAKPH, was selected from a disulfide constrained random heptapeptide library displayed on the pⅢ of phage M13 by panning against HBsAg purified from human plasma. The phage harboring the peptide was shown to bind tightly to HBsAg with a K_D of 2.9 nmol/L. The whole phage was then employed as a diagnostic reagent to establish a direct and an indirect phage-ELISA to detect HBsAg in HBV infected patients (Figure 5). Both assays were able to detect HBsAg down to 1 pg/mL. Most recently, the three-dimensional structure of this paratope mimic was elucidated by nuclear magnetic resonance (NMR) and demonstrated to interact with the "*a*" determinant of HBsAg[82]. These cumulative data prove that an antibody can be replaced by a short peptide and a phage harboring this peptide can be used as a sensitive diagnostic reagent.

Along the same lines, an M13 phage displaying a paratope mimic that interacts tightly with HBcAg was used as a diagnostic reagent to develop a phage-ELISA, dot blot

assay and immunoprecipitation assay[83]. The paratope mimic with the sequence WSFFSNI was isolated from a disulfide constrained random heptapeptide library by panning against the purified recombinant HBV capsid immobilized on a solid phage[84]. The recombinant phage with a K_D of 2.5 nmol/L, was able to detect a minimum amount of 10 ng HBcAg in both the phage-ELISA and dot blot assay. These assays can also be used to detect HBcAg released from the virion in HBV positive serum samples[83]. The sensitivity of the phage-based diagnostic assay was further improved by Monjezi *et al*[85] by combining TaqMan based real-time PCR and ELISA. The resultant phage display mediated immuno-PCR (PD-IPCR) is 10000 times more sensitive than the phage-ELISA.

The vast diversity of a combinatorial phage displayed peptide library also allows one to select epitopes by panning against antibodies, be it monoclonal antibodies or polyclonal antibodies from sera. The selected epitopes, on the other hand, can be used to diagnose antibodies in sera. The advantage of this strategy is that prior knowledge about a newly emerging etiological agent inducing disease-specific antibodies is not required.

Compared with phage displayed antibodies, the isolation of phage born peptides is faster, cheaper and less tedious as no immunization of animals is involved. The affinity of a selected peptide towards an antigen or antibody can be increased by displaying the peptide on the major coat protein of a bacteriophage, such as the pVIII in the filamentous phage. Apart from HBV, phage born peptides have also been employed extensively as diagnostic reagents for cucumber mosaic virus[86], Newcastle disease virus[87,88], *Salmonella enerica Serovar Typhi*[89,90], Nipah virus[91] and HIV-1[92]. All these remarkable examples demonstrate the potential of phage displayed peptides as diagnostic reagents for detecting etiological reagents and of course this can be further extended to detect an unknown microorganism.

DRUG DISCOVERY

HBV has killed millions of innocent people in this century and the previous one. Currently, there are about 370 million chronic HBV carriers worldwide, and their major hope is a rapid discovery of an effective treatment for HBV. Therefore, searching for a cure is no doubt will change human history. The introduction of mass vaccination of infants worldwide with HBV vaccines has significantly reduced the incident of liver cancer and chronic infection. Despite of effective vaccines against HBV, mutations of the viral genome have resulted in vaccine escape mutants. Treatments of HBV chronic carrier with nucleoside or nucleotide analogs have also given rise to mutants that are resistant to anti-viral drugs. Most frightening, HBV is now associated with HIV to threat the world. Treatments of patients co-infected with these two viruses, either by using drugs against one or both viruses, have led to the selection of resistance mutations. Therefore, there is always a continuing need for the discoveries of a new therapeutic agent to control HBV infection particularly for the newly emerging mutants.

Traditionally, drugs and therapeutic agents are discovered through isolation of active ingredients from medicinal plants or microorganisms following effective treatments of the illnesses with the crude extract of these sources. In modern drug discovery, structural bioinformatics allow *in silico* screening of chemical compounds that 'fit' the binding pocket of a targeted protein from virtual chemical libraries. The actual dynamic and physical properties of the *in silico* selected compounds always end in disappointment due to the difficulties in chemical synthesis, poor solubility, affinity and specificity, although, in some cases, the binding mechanism can be explained by structural analysis using X-ray crystallography and NMR. The emergence of phage display technology has offered a powerful and rapid method to identify novel peptides and antibodies from phage fusion peptide/antibody libraries that bind to wide range of antigens ranging from whole cells such as bacteria, parasites and viruses to macromolecules for instances proteins, carbohydrates and lipids. DNA recombinant technology enables peptides to be fused either to the major or the minor fusion coat proteins and eventually displayed on the surface of various bacteriophages[93]. Vast peptide libraries can be screened for specific ligands that bind a targeted protein through a biopanning process[94]. The isolated phages can be easily propagated by infecting *E. coli* and the peptide sequence can be deduced by nucleotide sequencing. The corresponding peptide can be chemically synthesized or produced using bacterial expression systems. Owing to the stringency multiple cycles of selection, the selected peptides always display high specific and affinity towards their targets, however for some peptides, stability and solubility are the main problems. Nowadays, peptide binding mechanism can be studied by structural approaches and binding assay using ELISA.

For the past two decades, phage-displayed combinatorial peptide libraries have been used widely to select anti-viral peptides against many viruses (see review in[95]). Similar to other anti-viral discoveries, the major processes in HBV life cycle are the potential targets for drug discovery. Specific ligands that bind the key players of the viral life cycle would interfere with HBV replication intracellularly or extracellularly. The potential extracellular targets include (1) neutralization of matured infectious virion; and (2) viral-host cell receptor interaction. The infectious viral particles can be neutralized easily by specific antibodies. By screening combinatorial phage display Fab fragment libraries against HBsAg, several groups have successfully isolated Fab fragments that neutralize infectious HBV particles[96-103]. In addition, the PreS1 region which possesses a dual function in the viral assembly and infectivity has also been targeted for anti-viral drug discovery by panning with phage display peptide libraries[81,104-107]. Intracellularly, HBcAg forms the viral nucleocapsid and interacts with the PreS region during the viral assembly, therefore, HBcAg is a common target for the

discovery of HBV inhibitors[84,108]. In addition to the intracellular inhibition of viral assembly, HBV release from the infected hepatocytes can be blocked by Fab fragments of intrabodies that could be selected easily from phage displayed antibody libraries. Intrabodies are intracellular antibody fragments that are expressed and functional inside a cell[109]. Several studies have shown that these phage derived intrabodies fragments bind immature HBV particles or proteins and potentially inhibit the viral secretion *in vivo*[78,110-112]. Targeting the extracellular steps of the viral cycle is relatively simpler and more straightforward compared to those of the intracellular steps, in which the anti-viral compounds must enter the membranous barrier of the liver cells. Nevertheless, selection of peptides against the association of HBcAg and L-HBsAg has been studied extensively and intensively, however, *in vivo* anti-viral activity has yet to be characterized in depth.

Intracellular targeting

HBcAg is an attractive target for the discovery of novel therapeutic agents. In the process of nucleocapsid formation, one copy of the viral pre-genomic RNA (pgRNA) is encapsidated in the cytoplasm[5]. Following the conversion of pgRNA to a mature viral genome (partially double stranded DNA), the nucleocapsid moves to the endoplasmic reticulum (ER) where it interacts with the ER membrane embedded with HBsAg which results in the envelopment of nucleocapsid. The mature virion is either secreted into the bloodstream or moved back to cytoplasm. This process is followed by disassembly of the nucleocapsid into individual HBcAg at the nuclear pore complex and the viral DNA is released back to the nucleus[113,114]. HBcAg has been shown to function as a transcriptional activator which enhances the viral replication[115]. The enrichment of HBcAg in this manner correlates with the high level of viral replication. Several anti-viral agents such as aptamers[116], heteroaryldihydropyrimidines[117], and single-chain variable fragment intrabodies[118] have been developed to target HBcAg. Using phage display technology, peptides were successfully selected from phage display peptide libraries to prevent its interaction with HBsAg[84,108] and Fab fragments of intrabodies neutralize HBcAg inside liver cells were also selected from phage display antibody libraries[78,110-112].

HBV was the first virus used in the selection of peptide inhibitors from a 6-mer linear phage display peptide library[108]. A phage bearing amino acid sequence LLGRMK that interacts specifically with the immobilized HBcAg particles with a K_D of 0.17 μmol/L was selected and the corresponding synthetic peptide ALLGRMK was shown to block the association of L-HBsAg and HBV nucleocapsid *in vitro*. Interestingly, neither peptide ^{21}LLTRIL^{27} of S-HBsAg nor GRMKG (C-terminal part of the selected peptide) inhibited the binding of L-HBsAg to HBcAg but this interaction was partially inhibited by the PreS1 amino acids ^{19}LDPAFR^{24}, the epitope of monoclonal antibody 18/7[108]. This indicates that the peptide mimics the internal region of HBsAg comprising distal amino acids which are brought into close proximity upon protein folding. Cell culture experiment also proved that the synthetic peptide was able to reduce HBV replication *in vivo* and the peptide binding site was later located to the tips of the spike that formed by amino acids 78-82 (^{78}DPASR^{82}) of HBcAg[119]. Mutations of D78 and E77 to an Alanine, in turn, dramatically reduced the binding affinity. Cross-linking study also proved that these residues are essential for the peptide-HBcAg interaction[120]. Although the 3-dimensional structure of the complex of HBcA-peptide was solved by cryo-electron microscopy, the detailed binding mechanism remains elusive due to its poor resolution[119]. In 2003, a modified biopanning approach using a disulfide-constrained heptapeptide library was employed. This approach has led to the isolation of cyclic peptide CWSFFSNIC and CWPFWGPWC exhibiting a higher affinity (> 10-fold higher than LLGRMK) towards HBcAg and similarly, the synthetic peptides blocked the association of HBcAg and L-HBsAg or monoclonal antibody C1-5, which has an epitope located at amino acids 78-83 of HBcAg, from interacting with its epitope[84]. These findings indicate that the linear and cyclic peptides bind different set of amino acids close to the tip of the spikes. The conformational constraint cyclic peptides were composed of mainly hydrophobic amino acids and it is believed that they interact with the hydrophobic amino acids located at the immunodominant region of HBcAg. Interestingly, the N-terminal half of peptide LLGRMK also contains hydrophobic amino acids, however it is unclear whether these residues interact with the same amino acids as the cyclic peptides. The 3-dimensional structure of the cyclic peptides in complex with HBcAg has yet to be identified. Further characterizations with the synthetic cyclic peptides were hampered by their hydrophobicity. Therefore these peptides need to be further modified in order to improve the solubility and bioavailability.

In 2009, Serruys *et al*[111] demonstrated for the first time that viral secretion in mammalian cells can be inhibited by intrabody-mediated inhibition. They constructed a single-domain antibody (VHH)-phage display peptide library and biopanned it against human plasma derived HBsAg. Up to 85% of the isolated phages are associated with S-HBsAg in relative to only 45% of the soluble VHHs expressed intracellularly. These intrabodies were shown to reduce HBsAg particles in plasma when they were expressed in the ER and shown to reduce the viral load up to 100-fold in a mouse model[111]. In the following year, similar approach was used by the same research group to isolate VHH intrabodies that bind HBcAg of *ayw* and *adw* subtypes[110]. As demonstrated in HBV transfected HepG2 cells, the nucleotropic targeted intrabodies reduced intracellular HBcAg, but interestingly, two out of six isolates increased the detection levels of intracellular HBeAg, which is likely due to HBeAg antigenicity displayed by the non-particulate HBcAg or retention of HBeAg inside the cells as a result of interaction with these intrabodies[110]. This hypothesis is in

good agreement with that of Walsh *et al*[112], who isolated intrabodies by screening a naive single variables domain (VNAR) display phage library against HBV precore antigen (preHBcAg) and proved that these intrabodies reduced the extracellular amount of HBeAg and intracellular preHBcAg[112]. HBeAg is believed to function as an immune tolerogen that could lead to the establishment of chronic infection[121]. Therefore, down-regulation of HBeAg should improve clinical outcome. In an earlier study, Tan *et al*[78] isolated single-chain variable fragment (scFv) against HBcAg from a phage display scFv library. Although the initial aim of this study was to develop an antibody system to detect HBcAg, expression of the ScFv inside liver cells particularly in the ER membrane could possibly convert the antibody fragments into potent therapeutic agents that could neutralize and inhibit viral secretion *in vivo*.

Extracellular targeting

The first step of HBV replication is the attachment of an infectious HBV particle with a receptor on the surface of hepatocyte. Molecules that mimic the structural elements of a host cell receptor could block subsequent infection steps. The major problems to study HBV infection are attributed to its narrow host range and the absence of a susceptible cell line for virus infection[122]. However, biochemical studies with purified components of duck HBV (DHBV) have revealed several candidates that involved in DHBV infection[123]. Several glycoproteins have been identified as potential DHBV receptors such as glycoproteins gp180/p170 that bind DHBV and *E. coli* derived GST-PreS polypeptides. These glycoproteins are later classified as the prototype members of membrane bound carboxypeptidase D (CPD)[124,125]. Urban and coworkers showed that DHBV PreS region binds duck CPD with a KD of 0.46 nmol/L[126] and also demonstrated that CPD plays pivotal roles for the virus entry and attachment *via* its carboxypeptidase-like (A and B) and non-enzymatic (C) domains, respectively[127]. Although these host proteins have been identified as the cellular receptor of HBV, identification of a promising inhibitor has yet to be reported. On the other hand, the PreS1 region that plays pivotal roles in the viral infectivity and assembly has been the main focus of many anti-viral studies using phage display technology[81,104,107].

Tan *et al*[81] biopanned the HBsAg (subtype *ad*) purified from human plasma with a disulfide constrained phage display peptide library and identified a predominant M13 pIII fusion heptapeptide CETGAKPHC that binds to the immunodominant region of HBsAg (amino acids 111-156) with dissociation constants 2.9 ± 0.9 nmol/L and 0.83 ± 0.63 nmol/L[81]. NMR study indicated that the peptide adopts *cis*/*trans* conformations due to Pro rotamerization and *in silico* analysis revealed two binding sites which correspond to the first (amino acids 107-137) and second (amino acids 138-149) loops of the immunodominant region[82]. This finding indicates that the peptide could be further developed as a therapeutic agent that blocks the entry of HBV into hepatocytes.

Instead of using the purified HBsAg from an infected human serum as a substrate for affinity selection, Deng *et al*[104] biopanned the thioredoxin tagged-PreS using an M13 pVIII 12-mer fusion cyclic peptide library. The recombinant PreS1 is more soluble and stable compared with the PreS region alone[105]. Following 5 rounds of biopanning, Deng *et al*[104] isolated peptides with a common sequence motif of W/FTXWW/F that interact with amino acids 21 to 47 of the PreS1 region. Biotinylated peptide bearing an amino acid sequence SGSGWTNWWST (amino acids SGSG is a linker) was able to precipitate the HBV particles produced by HepG2 cells[104]. Several other HepG2 proteins were also bound to the same PreS1 region[128,129]. Among others, homology search has identified a lipoprotein lipase; a key enzyme involved in the lipoprotein metabolism; which contains amino acid sequence SWSDDWWS at its C-terminal region and this sequence was conclusively shown to interact with the PreS1 amino acids 21-47 and HBV particles. Alanine mutation scanning of the tryptophan residues of this peptide abolished the binding of the PreS1 and HBV particles; however, these activities were retained if tryptophan was replaced with phenylalanine. The authors speculated that the tryptophan/phenylalanine may mediate interaction of protein and lipid/aqueous interface during the viral attachment due to its amphipathic character. Overall, this PreS1 binding peptide displays anti-viral activities by blocking the viral attachment to a hepatocyte and potentially reduces the viral infectivity. Independently, Wang *et al*[107] and Deng *et al*[106] isolated a dodecapeptide (KHMHWHPPALNT) and a disulfide-constraint heptapeptide (SRLLYGW), respectively, against the PreS1 region from phage display peptide libraries[106,107]. Although both peptides were shown to bind the PreS1 region and capture HBV virion produced by HepG2 cells, no sequence consensus was found between these peptides and also those isolated by Tan *et al*[81] and Deng *et al*[104]. Variability in the construction of phage display library and biopanning procedures may lead to identification of different peptides that might potently block viral-host cell interactions. Diversity of the selected peptide sequences indicates that various cellular proteins are involved during the viral attachment. Targeting these proteins particularly the cellular receptor could potentially reduce the viral infectivity *in vivo*.

EPITOPE MAPPING

HBV can be easily transmitted from asymptomatic carrier mothers to their newborn babies. Such perinatal transmission is commonly caused by an exposure to HBV contaminated blood during birth. To reduce the risk of such mother to baby transmission, passive immunization has been introduced in which babies born to HBsAg positive mothers are immunized at birth with hepatitis B immunoglobulin containing antibodies against HBsAg. These antibodies are effective in preventing HBV infection in infants who do not have sufficient time to develop their

own immune response. Apart from preventing perinatal transmission, these antibodies could also be used in acute exposure to blood containing HBsAg, sexual exposure to HBsAg, positive carriers and household exposure to people with acute HBV infection. After intramuscularly administration, the antibodies interact specifically with HBsAg epitopes and neutralize the virus. In other words, the paratopes of the antibodies interact with the epitopes on HBV. Antigenic epitopes are the region of an antigen that interacts specifically with an antibody or a T-cell receptor. In general, epitopes are divided into linear (continuous) and conformational (discontinuous) epitopes. A linear epitope is defined as a minimum stretch of continuous amino acids that are recognized by an antibody while the conformational epitope or mimotope are composed of discontinuous amino acids that brought into proximity as a result of 3-dimensional protein folding. A detailed study of epitope is the fundamental basis for the development of epitope-based vaccines, therapeutics, viral inhibitors and diagnostic reagents.

Various methods have been used to map the epitope of a specific antibody. Most of these methods rely on the ability of the antibody to recognize a linear or conformational epitope displayed on the surface of an antigen. The earliest method for epitope mapping known as the "pepscan method" was developed by Mario Geysen and coworkers in 1984[130]. With this method, a peptide library consisting of different lengths of overlapping oligopeptides covering an epitope is scanned over immobilized antibodies. Peptide array that interacts with the coated antibodies corresponds to certain aspects of the epitope. The major limitation of the pepscan method is that it only allows the identification of linear epitopes instead of conformational ones. X-ray analysis of antigen-antibody complexes is capable to identify the precise location of epitopes up to atomic resolutions, however, not all antigen-antibody complexes are readily crystallizable[131]. To date, about 70 co-crystals of antigen-antibodies have been solved by X-ray crystallography, demonstrating an exceptionally low efficiency of this method[132]. On the other hand, NMR is an alternative approach to achieve similar purpose but this method is limited to antigen-antibody complexes with molecular weight less than 30 kDa[133]. Nevertheless, NMR provides the thermodynamic characteristics of antigen-antibody complexes in solution. Overall, the accuracy and precision of the above mentioned biophysical approaches towards epitope mapping are restricted by the tedious and laborious techniques and the requirement of advanced instrumentation and expertise.

In 1990, Scott and Smith[94] demonstrated that epitope mapping can be accomplished easily by screening a random peptide library against sera or monoclonal antibodies that immobilized on a solid surface. Epitope mapping using phage display technology offers the advantages of (1) identifying both continuous and discontinuous epitopes; (2) independent of advanced instrumentations; (3) cost effective and simple experimental protocol; and (4) exploring the variety of binding sequences by screening with phage display peptide libraries containing fusion peptides with different lengths or conformations such as linear or disulfide-constrained fusion peptides. Precise epitope mapping of sequential or conformational epitopes recognized by monoclonal or polyclonal antibodies is important to understand the mechanism of immune response, host-virus interactions and development of vaccines and diagnostic tools. Such mapping requires the identification of shortest amino acid stretches within the polypeptide that is still capable to bind to the target antibody.

Generally, immunological studies have identified three HBV antigens to be clinically relevant: (1) HBsAg; (2) HBcAg; and (3) HBeAg. HBsAg has been extensively used to induce protective immune response in immunized individuals while HBcAg and HBeAg have served widely as diagnostic markers. Due to the vaccination value, be it an active or passive immunization, most of the early epitope mapping of HBV proteins was focused on the PreS regions particularly the PreS1 region that associates with the viral host cell receptors. With the development of phage display technology, epitopes of various antibodies raised against the PreS1[96,134,135], PreS2[136] and S regions[137-140] have been mapped by screening phage display peptide libraries. The strategies and outcomes of the epitope mapping are summarized in Table 2, and reviewed critically in the following sections.

Epitope mapping of the PreS1 region

Germaschewski and Murray[134] biopanned the first established monoclonal antibody MA18/7 that interacts specifically with the PreS1 region. Following four rounds of biopanning, phage bearing pⅢ fusion hexapeptides with amino acid sequence motif LDPX (F/Y), in which, X represents small side-chain amino acids such as alanine, glycine or valine were selected. The dipeptide DP was conserved from rounds 2 to 4 among all the analyzed phagotopes. This sequence motif mimics three or four residues of ^{19}LDPAF^{23} of the PreS1. Recently, the 3-dimensional structure of monoclonal antibody MA18/7 in complex with epitope ^{19}LDPAF^{23} fused to HBcAg immunodominant region was determined by cryoelectron microscopy[141]. Interestingly, this short amino acid sequence was also identified as the receptor-binding domain of L-HBsAg although similar motif was also found in some bacteria and cellular adhesion molecules[133].

This conserved motif was also reported when a 15-mer phage display peptide library was biopanned against the monoclonal antibody MA18/7 that had been immobilized on polystyrene beads[135]. Diversity of the longer flanking amino acid sequences around the tetramer [DPX(F/Y)] suggests that these adjacent amino acids do not contribute to specific binding. Alignment of the selected sequences with the PreS1 amino acids 28-35 (^{28}HQLDPAFGAN^{35}) indicates that all the phagotopes contained the core motif DX$_1$X$_2$F, with X$_1$ constituted of either proline or arginine although leucine and serine occurred once each and X$_2$ encompassed by small side-chain residues such as valine, alanine, glycine and serine,

Table 2 Epitope mapping of anti-hepatitis B virus antibodies

Antibodies against	Phage library used	Epitopes	Remarks	Ref.
PreS1 region				
MA 18/7	M13 pⅢ 6-mer	ALDPAY, SLDPGF, PDPGFN, QLDPGF	The dipeptide DP was conserved from rounds 2 to 4 among all the analyzed phagotopes. Selected peptides were mapped to amino acids 19-23 of the PreS1 region	Germaschewski *et al*[134]
MA 18/7	M13 15-mer liner	SDTRGDPVFNLPFQ APVDSVFDRAFSAYL	Isolated peptides contain motif similar to LDPAF	D'Mello *et al*[135]
MA BX-182	M13 pⅢ 12-mer linear	SVPPPHTRSASG	Peptide matched amino acids ^{17}SVPNP^{21} of the PreS1	Zhang *et al*[144]
	M13 pⅢ 7-mer disulfide constrained	CTNPVLRSC	Peptide spatially matched some amino acids within the same region but in a C- to N-terminal direction	
PreS2 region				
HBsAg polyclonal sera	M13 pⅢ 12-mer linear	TANGFYRLPSGS	Peptide sequence mapped to amino acids 135-146 of the PreS2 region	Zhang *et al*[136]
S-region				
MA RFHBs6	M13 pⅢ 15-mer linear	TSNTHAC(R/K)TCSNPSR	The peptide sequence is similar to amino acids 115-129 of the S region	Motti *et al*[140]
MA BA1	M13 pⅢ 15-mer linear	PHDGNRAFPRTKVTM HMPRDANRHHQHPST SSLGSDHNARWVKRF	Low sequence homology with the S region	
MA 6H6B6	M13 pⅢ 12-mer linear M13 pⅢ 6-mer linear	WPHNWWPHFKVK QGFLPQ	Peptide sequence homology not found Peptide sequence mapped to amino acids 101-105 of the S-region	Jolivet-Reynaud *et al*[137]
MA H166, H5, H35, H53	M13 pⅢ 30-mer linear	[1]Peptide Xn-CXTC-Xn	Sequence motif of CXTC was mapped to amino acids 121-124 of the S-region	Chen *et al*[138]
IgG derived from S-HBsAg immunized chimpanzee	M13 pⅢ 6-mer linear	TRVPRR, SRLPLR, SRLPKR	Sequence motif SxxPxR was mapped to amino acids 117-122 of the S-HBsAg	Germaschewski *et al*[139]

[1]x_n represents random flanking amino acid sequences. Position X is constituted either by arginine, lysine or valine. MA: Monoclonal antibody; HBsAg: Hepatitis B surface antigen; IgG: Immunoglobulin G.

which is consistent with Germaschewski and Murray's[134] earlier finding. The biopanning results either using 6- or 15-mer phage display peptide libraries are consistent with those of epitope mapping by using the bidirectional shortening of the PreS1 with exonuclease digestion, which conclusively defined the minimal epitope of the PreS1 to be DPAF[142]. Searching through the Protein Databank (PDB) revealed that the amino acid sequence DPAF adopts either a β-turn or as part of an α-helical structure. The main-chain carbonyl of the aspartate is always hydrogen bonded to the main-chain nitrogen of phenylalanine[143]. Therefore, it is not surprising that the alanine cannot be replaced with amino acids bearing bulkier side-chains.

In a separate study conducted by Zhang *et al*[144], a monoclonal antibody raised against HBsAg of *adw* subtype (monoclonal antibody BX-182) was used to select phagotopes from two different random peptide libraries. An early study demonstrated that a monoclonal antibody preferentially neutralized infectivity of HBV *adw* subtype in a chimpanzee model and this prompted the authors to map the neutralization epitope of *adw* HBsAg. Initial affinity selection using a disulfide-constrained heptapeptide library successfully identified phage bearing a cyclic heptapeptide CTNPVLRSC. In a separate experiment, affinity selection with a 12-mer phage display peptide library identified a linear dodecapeptide SVPPPHTRSASG. Sequence homology search showed that part of the dodecapeptide (SVPPP) matched the N-terminal region of the PreS1 between amino acids ^{17}SVPNP^{21} with a replacement of proline with asparagine. On the other hand, the disulfide-constrained loop of CTNPVLRSC also spatially matched some amino acids within the same region but in a C- to N-terminal direction, indicating that ^{18}VP^{19} are the core amino acids for monoclonal antibody BX-182 binding as they are conserved in both isolated sequences. Sequence homology search also revealed that the Val/Pro motif is conserved among genotypes A, B, C, F and H of *ad* subtype but it is absent in most genotypes of *ay* subtypes or in genotypes D, E, and G of *ad* subtype, in which these antigenic residues are replaced with Thr/Ser, Thr/Thr or Ala/Ser, respectively. This result indicates that the binding of monoclonal antibody BX-182 is subtype-specific and the isolated peptide can be used to discriminate *ad*/*ay* subtypes in the diagnosis of potential HBV escape mutants.

Epitope mapping of the PreS2 region

The PreS2 region possesses significant clinical importance and it contains T-cell and B-cell epitopes which confer protection by inducing strong immune response in immunized chimpanzees[145,146]. To our knowledge, epitope mapping against the antibodies that recognize the PreS2 region was scarcely reported particularly with

phage display peptide library. This is most probably due to its short amino acid sequence comprises only 55 amino acids. Nevertheless, Zhang *et al*[136] biopanned a phage display library of dodecapeptides against the HBsAg polyclonal human sera. Following 3 rounds of affinity selection, phage bearing the fusion pⅢ peptide TANGFYRLPSGS; which mapped to amino acids 135-146 (^{135}RVRGLYLPAGGS^{146}) of the PreS2 region; was selected. This region was previously proved to be the antigenic epitope of monoclonal antibody 25-19, which has been shown to display neutralizing and protective activities[147,148]. Several epitope mappings of the PreS2 region were conducted by using the pepscan method[149-152].

Epitope mapping of the S region

Two antibodies namely monoclonal antibodies RFHBs6 and BA1, were pooled from HBV chronic carriers and used as targets for biopanning using a 15-mer phage display peptide library[140]. Single round of affinity screening against the monoclonal antibody RFHBs6 successfully isolated phage carrying pⅢ fusion peptide TSNTHAC(R/K)TCSNPSR, which is strikingly similar to amino acids 115-129 of the S region (^{115}TTSTGPCKTCTTPAQ^{129}). The two cysteine residues of the peptide are believed to be disulfide bonded and antigenically mimicking the natural epitope. On the other hand, similar approach was applied to isolate phagotopes that recognize monoclonal antibody BA1. Three peptides bearing the amino acid sequences PHDGNRAFPRTKVTM, HMPRDANRHHQHPST and SSLGSDHNARWVKRF were identified. Multiple sequence alignment indicated a low sequence similarity among these sequences with amino acids 127-135 at the N-terminal region of S-HBsAg (^{127}PAQGNSMFP^{135}). Interestingly, phagotopes selected against monoclonal antibodies RFHBs6 and BA1 did not cross-react with their counterpart's target, indicating that the antigenic determinants are distinct.

Epitope mapping of monoclonal antibody 6H6B6 which recognizes specifically the S-HBsAg was carried out by biopanning the 6-mer and 12-mer phage display peptide libraries[137]. A selected phage bearing the fusion dodecapeptide WPHNWWPHFKVK did not share similarities with the primary sequence of the S-HBsAg as well as the PreS2 sequence but reacted with the monoclonal antibody 6H6B6, indicating that the selected phagotope mimics the epitope conformation. In contrast, biopanning with a 6-mer phage display peptide library had selected a pⅢ fusion hexapeptide QGFLPQ that shared 4 identical amino acids localized between amino acids 101-105 (^{101}QGMLP^{105}) of S-HBsAg. Overlapping peptides (^{99}DYQGMLPVCPLI^{110} and ^{102}GMLPVCPLIP^{111}) corresponding to this region blocked the binding of monoclonal antibody 6H6B6 to the phage bearing pⅢ fusion peptide QGFLPQ. Furthermore, peptides coated on an ELISA plate were not recognized by monoclonal antibody 6H6B6, suggesting that amino acids 101-105 is part of a conformational epitope that corresponds to the N-terminal region of the major hydrophilic domain of the S-HBsAg. Peptide affinity selection against monoclonal antibodies 27E7F10 and 2G2G10 isolated highly hydrophobic peptides which were localized to amino acids 214-219 and 199-208, respectively, of the S-HBsAg. Antibody-peptide competition assay, however, was not carried out due to hydrophobicity of the flanking peptide sequence.

Affinity selection using a 30-mer phage display peptide library against monoclonal antibody H166 identified phagotope bearing a sequence motif CXTC, which localized to residues 121-124 of the S-HBsAg[138]. Coincidently, in the same year, Germaschewski and Murray[139] selected hexapeptides that bound to serum IgG derived from the S-HBsAg immunized chimpanzee. This 'shotgun' approach selected 20 different pⅢ fusion hexapeptides with some common motifs that match groups of 3 or 4 amino acids along the S-HBsAg. In an additional round of selection (micropanning using a phage sandwich ELISA), the number of tight binders had been reduced to 7 with pⅢ fusion peptide bearing the motif SxxPxR, which corresponds to amino acids 117-122 of the S-HBsAg (^{117}STGPCR^{122})[139]. It is believed that the two cysteine residues within this region cyclized to form a disulfide-constrained loop conformation that resembles the native structure of HBsAg. Biopanning with other monoclonal antibodies (H5, H35 and H53) also identified fusion peptides flanking by cysteine residues that are separated by 2 to 15 amino acids[138]. Some selected peptide contained two separate regions that matched the S-HBsAg sequence, indicating that these segments are brought together into a close proximity as a result of protein folding.

DRUG AND GENE DELIVERY

HBV carriers have an increased risk of developing liver cancer or HCC. Worldwide, HCC is the fifth most common cancer that contributes to 5.6% of all human cancers and ranks third among cancer-related mortalities[153]. Recently, gene therapy has become a promising approach to treat cancers and virus-based nanoparticles, particularly bacteriophages, have been proposed as gene and drug delivery vectors.

Filamentous bacteriophages have been at the cutting edge of new developments in nanotechnology which create innovative applications in gene and drug delivery to target specific cell types. The M13 phage particle has a diameter of about 6 nm and about 900 nm long, which looks like a long thread under an electron microscope. This flexible nano-scale filament has been exploited as a template for the synthesis of semiconducting nanowires and lithium ion battery electrodes[154]. Several studies have shown that filamentous single-stranded bacteriophages can transfer genetic materials into mammalian cells in the presence of DEAE dextran and lipopolyamine[155]. In 1994, Hart *et al*[156] demonstrated that filamentous bacteriophages can be targeted to the cell surface and shown to be internalized with peptides. Larocca *et al*[157] showed that bacteriophage harboring the gene for growth fac-

tors are capable of delivering the gene into mammalian cells. Phages are potential gene and drug delivery systems for several main reasons. They lack tropism for eukaryotic cells; therefore phage vectors are safer than animal viruses. They are also physically stable and can be produced in large amount at lower cost. Besides, they can be cultured easily up to industrial scale, their coat proteins protect internal genetic materials from cellular degradation and they are physically stable within a wide range of temperature and pH[158]. Before a phage can be used as a drug or gene delivery vehicle for human, the specificity of the phages towards bacteria in human must be studies in depth. This could prevent or reduce the change of microbiome patterns in a healthy individual.

An interaction between HBV envelope protein, particularly the PreS regions, and a specific cell surface receptor is believed to be the initial step of HBV infection through attachment to hepatocytes. In order to develop a gene delivery system, the PreS regions were displayed on the pⅢ protein of phage M13[41] and used to transfect human hepatocarcinoma cell line HepG2[159]. The recombinant phage, namely M13-PreS, was shown to transfect HepG2 cells but the efficiency was very low, which could be attributed to the low valency of the displayed ligands. In order to improve the level of transfection, two fragments of the PreS1 region, residues 1-47 and 60-108, were fused separately to the 10B protein and 415 copies of these fragments were successfully displayed on the surface of phage T7. The recombinant phage displaying the amino acids 60-108 was shown to be the most effective in transfecting and internalizing into HepG2 cells in a dose- and time-dependent manner[160]. The capability of the phage to transfect HepG2 cells indicates the potential of the phage display system as a gene or drug delivery system for HCC. In an *in vivo* study, Zhang *et al*[161] further demonstrated that a filamentous phage bearing the amino acid sequence FQHPSFI bound to tumor cells following intravenous injections into BALB/c mice. This reaffirms the potential of phage display as a drug or gene delivery system to target HCC.

A combination of phages and other virus-like particles, for instance HBV capsid, has broadened their applications in gene and drug delivery. HBV capsid is made of 240 or 180 copies of HBcAg which form large and small icosahedral structures with triangulation number $T = 4$ or $T = 3$, respectively[162]. For the past 30 years, HBV capsid has been used extensively as molecular carrier for foreign epitopes in the development of multi-component vaccines (for reviews, see[33,34,163]). In addition, Lee *et al*[164] demonstrated that HBV capsid produced in *E. coli* can be dissociated into dimers by urea and guanidine hydrochloride (GdnHCl) and reassembled when the denaturing agents were removed by dialysis. This feature allows the capsid to package molecules in its empty inner cavity and can be further developed into a vehicle for delivering therapeutic molecules into a targeted organ. Cryoelectron microscopy[162] and X-ray crystallography[165,166] revealed that both the large and small HBV capsids are spiky and each spike is formed by four α-helix bundles, two from each HBcAg monomer. Affinity selection of HBV capsid with a hexapeptide phage display library, has successfully isolated peptides with the core motif LLGRMK[108]. Subsequently, the binding site of peptides containing this motif sequence was located precisely at the tip of the spike of HBV capsid by using cryoelectron microscopy[119] and mass-spectrometry[120]. A 12-mer peptide GGGSLLGRMKGA containing this motif sequence was designed as a "nanoglue" to display cell-internalizing peptides (CIP) of HeLa cells on the surface of HBV capsid[167]. Astonishingly, the HBV capsid delivered its cargo, be it oligonucleotides or fluorescein molecules into HeLa cells specifically[167]. Thus, a combination of phage display, viral-like particles (VLPs) and the nanoglue concept creates innovative applications, not only to combat HBV, but other diseases.

CONCLUSION

In general, mass immunization of world population with the recombinant HBV vaccine produced in yeast is an enormous success. HBV prevalence has now dropped dramatically and of course countless lives have been saved. For pharmaceutical industry, the recombinant vaccine has grown into a multi-billion-dollar business. Initially the market was monopoly by two giant pharmaceutical companies, but now many biotechnology companies are mushrooming and penetrating into this industry. Are these newly established companies employed the conventional technology to produce the same vaccine or are they going to invest and introduce a novel vaccine which is cheaper and more effective? If innovation is the only solution, phage display provides an alternative means for scientists and entrepreneurs to invent and introduce a novel HBV vaccine into the market. The strategies that can be used to achieve this aim are not only limited to; (1) display of immunogens on phage particles; (2) identification of epitope or mimotope mimics from sera; and (3) phage-delivered DNA vaccine. Although, the phage-based immunogens yielded promising results in animal immunizations, could they survive the stringent test of the time (about 10 years) and cost of clinical trials as well as product development, which would at least amount to about US$ 300 million? Economically, can they compete with the existing effective recombinant vaccine?

HBV is commonly used as a proof-of-concept target for novel diagnostic technologies due to its threat on global public health. The ultimate aims of these new technologies are: (1) to attain the highest sensitivity and specificity in the shortest time; (2) to obtain as much information as possible in a single test with the cheapest cost; and (3) to achieve simplicity. For the detection of HBV antigens in sera by using ELISA, the type of antibody mostly used is the IgG molecule, either raised in animals or produced *via* hybridoma technology. The IgG is a relative large and complex molecule with disulfide bonds and glycosylation which reduce its stability during

storage and transportation. In addition, its production is laborious, time consuming and costly. These limitations can be overcome by phage display technology which allows the antigen recognition fragments of antibodies to be engineered and selected from a combinatorial library. Many studies have demonstrated the potential of phage display antibodies and peptides as diagnostic reagents for detecting HBV serological markers. Most recently, we have proven that phage-ELISA can be combined with real-time PCR for the detection of HBV antigen.

Phage display technology offers the opportunities to display vast number of peptide sequences on the surface of the minor or major coat proteins of bacteriophages. Affinity selection through biopanning has led to the identification of ligands that interact with the target molecules. Using liver cell lines, anti-viral activities of the isolated peptides/antibody fragments were demonstrated to inhibit intracellular and extracellular steps of HBV life cycle and subsequently reduced the HBV replication rate significantly. To date, most of the isolated therapeutic agents and their derivatives are targeting the viral proteins particularly the PreS1 region and HBcAg instead of the host cell proteins, although several authentic host proteins were discovered. These host cell proteins involved directly or indirectly in the viral attachment and penetration. It is believed that a set of periplasmic and cytoplamic proteins is involved in a cascade of reactions that lead to the internalization of the viral particles. In addition to current anti-viral therapeutics, discovery of therapeutic agents targeting the host cell proteins involved in the viral infection process is another potential strategy to combat HBV replication and infection.

The most heavily mapped HBV protein is the PreS1 region due to its clinical importance in vaccine design, diagnostic and therapeutic purposes. The S-HBsAg was also mapped considerably due to its abundances in the serum of HBV infected individuals. Although HBcAg is important for diagnostic purposes, its epitope has not been mapped extensively particularly with phage display technology. This is partly due to the availability of the 3-dimensional structure of HBcAg capsid which provides structural basis for the understanding of antibody-antigen binding. The epitopes of HBcAg capsid have been mapped extensively and precisely by examining the complex of core shell with antibodies using cryo-electron microscopy[168-171]. In contrast, the 3-dimensional structure of HBsAg has not been elucidated, probably due to its heterogeneity owing to high lipid content and extensive disulfide linkages. Thus, biophysical determination of epitope with other means is essential to map the epitope of HBsAg. Without the 'golden standard' mapping approaches such as X-ray crystallography, cryo-electron microscopy and NMR analysis, the pre-defined peptide epitopes have to be validated by various means which include mutagenesis, peptide scanning, cell culture and animal immunization. Furthermore, interaction between the phages and the antibody provides a means in terms of physical dynamics, which is greatly affected by physical parameters such as pH, temperature, ionic strength, and the peptide sequence polarity. It is common that a synthetic peptide alone behaves differently from the corresponding phage fusion peptide. Optimization of the internal or flanking sequence, sometime, is required to improve the binding efficacy.

The ultimate aim of drug or gene delivery is efficient conveyance of bioactive compounds to a specific target. The activity of the delivered substance is maintained for a desired length of time. The delivery vehicle must be biologically stable under physiological conditions. The packaging materials must be carefully chosen in order to protect the cargo. HBcAg with its unique capability to self-assemble into VLPs under permissive conditions is an excellent packaging material for foreign substances. Denaturing and renaturing of HBcAg VLPs allow therapeutic molecules to be packaged and protected inside the VLPs. These therapeutics molecules can be targeted specifically to an organ or a tissue by displaying CIPs on VLPs *via* the nanoglue concept. Nevertheless, the stability, solubility, specificity, and bioavailability of the nano-delivery vehicles and their therapeutic compounds must be studied thoroughly.

The innovative applications of phage display in epitope mapping and the development of vaccines, therapeutic agents, diagnostic reagents, as well as gene and drug delivery systems are not limited to HBV, they can be further extended to other microorganisms.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (9): Hepatitis B virus

Bacteriophages and their applications in the diagnosis and treatment of hepatitis B virus infection

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Abstract

Hepatitis B virus (HBV) infection is a major global health challenge leading to serious disorders such as cirrhosis and hepatocellular carcinoma. Currently, there exist various diagnostic and therapeutic approaches for HBV infection. However, prevalence and hazardous effects of chronic viral infection heighten the need to develop novel methodologies for the detection and treatment of this infection. Bacteriophages, viruses that specifically infect bacterial cells, with a long-established tradition in molecular biology and biotechnology have recently been introduced as novel tools for the prevention, diagnosis and treatment of HBV infection. Bacteriophages, due to tremendous genetic flexibility, represent potential to undergo a huge variety of surface modifications. This property has been the rationale behind introduction of phage display concept. This powerful approach, together with combinatorial chemistry, has shaped the concept of phage display libraries with diverse applications for the detection and therapy of HBV infection. This review aims to offer an insightful overview of the potential of bacteriophages in the development of helpful prophylactic (vaccine design), diagnostic and therapeutic strategies for HBV infection thereby providing new perspectives to the growing field of bacteriophage researches directing towards HBV infection.

Key words: Bacteriophage; Hepatitis B virus; Phage display; Phage library; Biopanning; Diagnosis;Treatment; Vaccine development

Core tip: Gaining insight into the role played by bacteriophages, viruses with prokaryotic hosts, in the development of helpful diagnostic and therapeutic approaches for hepatitis B virus (HBV) infection is of paramount importance. Natural presence of bacteriophages in the human body, as a major constituent of the gut flora, creates new opportunities in directing the developed approaches towards HBV infection. Undoubtedly, exploitation of this hidden potential of bacteriophages paves the way for introduction of novel methodologies for the detection and therapy of prevalent infection of HBV.

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INTRODUCTION

Hepatitis B virus (HBV) infection constitutes a significant health challenge worldwide and is considered as one of the most prevalent chronic viral infections in human. Chronic hepatitis can lead to a variety of liver disorders such as cirrhosis and hepatocellular carcinoma (HCC). HCC, one of the most common malignancies, is a leading cause of cancer-associated death in the world[1]. Human HBV belongs to the Hepadnaviridae family of viruses and human and higher primates are known as

Table 1 Names of the seven distinct proteins encoded by four overlapping genes contained in the genome of hepatitis B virus

	List of HBV proteins
Large surface antigen	L-HBsAg
Medium surface antigen	M-HBsAg
Small surface antigen	S-HBsAg
Core antigen	HBcAg
Generated by proteolytic processing of the pre-core protein (non-structural protein)	HBeAg
Reverse transcriptase activity	DNA polymerase
Unknown function (non-structural protein)	HBx protein

HBV: Hepatitis B virus; HBsAg: Hepatitis B virus surface antigen; HBcAg: Hepatitis B virus core antigen; HBeAg: Hepatitis B e antigen.

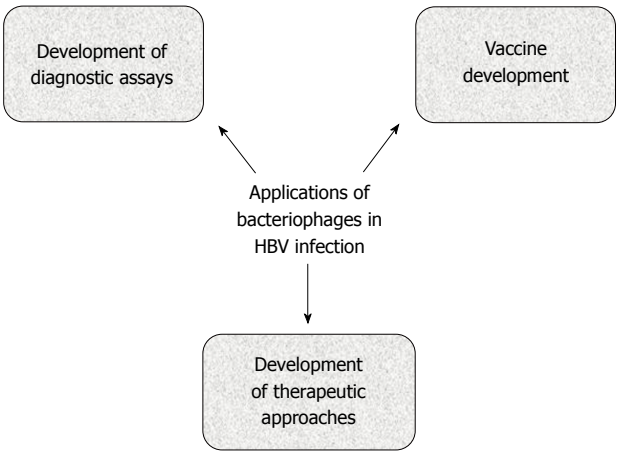

Figure 1 Various potentials of bacteriophages for hepatitis B virus infection.

the exclusive hosts for viral infection. The intact virion is composed of viral genome that is a 3.2 kb partially double-stranded circular DNA - enclosed in a nucleocapsid - and an outer layer. The nucleocapsid is made up of a large number of core antigen (HBcAg) molecules residing in the internal section of viral envelope. The outer layer contains surface antigen (HBsAg) molecules situated at the external portion of viral envelope. There are three different but related forms of HBsAg including large (L), middle (M) and small(S)-HBsAg. HBsAg harbors sets of epitopes known as immunodominant region. These highly conformational epitopes are positioned within a double-looped structure and trigger protective antibody responses in human. The antigenicity of immunodominant region can be used for detecting HBsAg. PreS1 (presurface 1) region - with 108 or 119 amino acids dependent on serotype - in the longest surface antigen (L-HBsAg) and on the outermost division of viral surface has been demonstrated to be implicated in the interaction of viral envelope with a specific receptor on the surface of hepatocytes. This HBV-hepatocyte attachment is known to be the initial step of viral infection. Furthermore, PreS1 region has been reported to play roles in the assembly and budding of virions[2-4]. Given the importance of PreS1 in viral assembly and infectivity, development of reagents with great affinity and specificity to this region of HBsAg is of particular importance for both diagnosis and treatment of HBV infection.

In general, HBV genomic DNA has four overlapping open reading frames that encode seven distinct proteins (Table 1). DNA polymerase, located within the nucleocapsid, is covalently attached to viral double-stranded DNA. Covalently closed circular DNA (cccDNA) which functions as the chief transcriptional template makes an essential contribution to the durability of infections caused by HBV and persists as an episome in HBV-infected hepatocytes. It can remain following antiviral therapy and even following apparent elimination of viral infection[5,6].

Currently there exist various diagnostic and therapeutic approaches for HBV infection. However, prevalence and also threatening consequences of HBV chronic infection emphasize the need to develop novel methodologies for the prevention, diagnosis and treatment of viral infection. Within the recent several years, numerous reports have revealed the potential of bacteriophages in this regard. These studies have shed light on the fact that one category of viruses called bacteriophage (with prokaryotic hosts) can serve as powerful tools to take prophylactic, diagnostic and therapeutic measures towards another category of viruses called hepatitis B virus (with eukaryotic hosts). The current review tells the tale of these two viruses. This review aims to provide opportunity for readers to gain a novel insight into the potential of bacteriophages in the emergence of new approaches for the prevention, detection and treatment of HBV infection. To this end, we discuss various applications of bacteriophages in three areas related to HBV infection. These areas include development of bacteriophage-based diagnostic tools, bacteriophage-based treatment modalities, and bacteriophage-based vaccines for HBV infection (Figure 1). In view of the paramount importance of phage display concept in presenting numerous bacteriophage-mediated diagnostic and therapeutic platforms for HBV, we address in detail phage display technology and phage display libraries and also provide description to various aspects of this powerful technology in order to formulate helpful diagnostics and therapeutics for HBV infection.

BACTERIOPHAGES AS TOOLS FOR MEDICAL PURPOSES

Bacteriophages are naturally-occurring viruses that specifically infect bacterial cells. These prokaryotic viruses are the most abundant life forms in the biosphere. In comparison with bacterial cells, the number of phage virions is 10 fold higher and estimations of phage frequency approximate their number to 10^{30} particles. They can be tracked down in an innumerable variety of environments ranging from oceans' depths to hot springs. One of the most attractive habitats of phages is the body of human and animals particularly their gastrointestinal tract[7,8]. Di-

gestive system of human is home to a huge number of phage particles. These phages, along with their bacterial hosts, hold a major role in forming the gut flora. Interestingly, phages exhibit tremendous diversity in a manner that a large number of newly sequenced phage genes are lacking recognized homologous counterparts deposited in databases[9]. This is a reflection of the fact that the phage world is a terra incognita and many regions of this vast territory remain a mystery.

Structurally, bacteriophages harbor DNA or RNA as their genetic material which is surrounded by a protein coat or capsid. Being metabolically inert, bacteriophages take advantage of their host bacterial cells for growth and amplification. Based on the life cycle, they can be placed in two categories of lytic and lysogenic. Lytic or virulent phages multiply within the bacterial host, and subsequently release their assembled particles through lysis of the host cell. On the contrary, lysogenic or temperate phages integrate their genetic material into the host chromosome leading to their replication together with the host bacterium for generations[10]. In response to harsh conditions such as ultraviolet (UV) irradiation, the genome-residing prophage can undergo induction and change into lytic phage.

When tracing back to the era of emergence of molecular biology, it becomes clear that from the very beginning bacteriophages have played a leading role in the field. They have made an essential contribution to solving many fundamental questions of molecular biology and genetics. With the elapse of time, this role has expanded and triggered developments in a variety of biotechnological and medical areas. Some of these achievements have revolutionized the view of researchers towards biological issues. The ongoing advances in bacteriophage research have attracted tremendous attention to the potential applications of these bacterial viruses in the clinical context. These progresses have paved the way to use bacteriophages for the prevention, detection, and treatment of various pathological disorders.

PHAGE DISPLAY

Bacteriophages indicate a high level of genetic flexibility. This characteristic established ground work for the formulation of phage display methodology. The initial concept of phage display was presented in the pioneering work of George P Smith conducted at the University of Missouri in 1985[11]. In this molecular selection technique a foreign DNA fragment is inserted into the gene encoding one of the phage coat proteins. The DNA-encoded peptide/protein is then displayed as fusion to the coat protein on the surface of phage particle, where it is available to be involved in binding interactions with target molecules. In this manner, a physical linkage is formed between the surface-expressed peptide/protein and the DNA that encodes it. This genotype-phenotype connection is one of the most significant aspects of phage display[12,13]. This technique has served to display a variety of molecules such as short peptides, individual protein domains, whole proteins, antibody fragments, receptors and enzymes on the surface of phage particles[14-16]. It is now well established that phage display as a robust and powerful technology represents huge potential to be used in a wide spectrum of biomedical and pharmaceutical areas including drug discovery, vaccine development, isolation of cell/tissue/disease-specific biomarkers (*e.g.,* a variety of cancers), protein-protein interactions, receptor-ligand characterization, gene/drug delivery and targeting, bioimaging and biosensing, neurobiology, proteomics, functional genomics, and antiviral research[17-25].

All five structural capsid proteins of M13 phage have the capacity to be exploited for expression of heterologous (poly)peptides. Each of the coat proteins has its own benefits and pitfalls. However, the most frequently used proteins for this purpose are minor coat protein (p Ⅲ) and major coat protein (pⅧ). pⅢ is a 400 amino acid-long protein implicated in binding of the phage particle to the host bacterium during infection. pⅧ with its 50 amino acids is recognized as the most abundant protein of M13 (about 2700 copies per phage virion) and plays a very critical role in shaping the long filamentous morphology of the phage. Generally, pⅧ is suitable for display of smaller proteins and pⅢ is proper for display of larger proteins. On the other hand, the abundance of pⅧ on the phage surface makes it possible for display of a higher number of protein of interest. But, pⅢ has fewer copies (3-5 copies) on the phage surface and is appropriate for display of a less number of protein of interest[26,27]. Other proteins of M13 are less matched for display purposes. This impaired capability for efficient display is due to the lack of full accessibility of these proteins in the context of intact phage particles. For example, some parts of pⅥ and pⅦ are not accessible in intact phage. Furthermore, the adverse effects of the displayed peptide/protein during phage assembly can, to some extent, result in low efficiency of display[28]. Choosing the coat protein (as fusion partner) on which the peptide or protein of interest will be displayed depends upon the type of the displayed ligand as well as the objective which is pursued.

Various bacteriophages such as filamentous phages, lambda, T4 and T7 have found application for surface display of foreign ligands. Platforms based on M13 (as a filamentous bacteriophage) constitute the most-widely used bacteriophage-based display tools. Filamentous bacteriophages (M13, f1 and fd) are rod-shaped bacterial viruses that are assembled at the membrane of the host bacterium where the phage genome is extruded through membrane pores. When assembled, the phage particle is composed of a single stranded, circular DNA enclosed in a proteinaceous coat with five different coat proteins[13,29]. Filamentous bacteriophages represent some advantages to be used as cloning vehicles and phage display technology platforms. Their genome can tolerate insertion of large DNA fragments in the nonessential regions without disrupting phage packaging. This high degree of adaptability is reflected by the fact that an increase in the

genome size is accompanied by a parallel increase in the length of the phage particle as well as the number of p Ⅷ molecules. Furthermore, M13 genome can be isolated as both single and double stranded forms. On the other hand, non-lytic multiplication of M13 phage leads to the accumulation of high concentrations of phage particles in the infected bacterial host[13,26].

PHAGE DISPLAY LIBRARIES

Combinatorial chemistry has proven to be a growingly potent and insightful methodology in modern drug development. This basic discovery technology can be used to generate combinatorial chemical libraries. In a general sense, combinatorial libraries are a diverse repertoire of molecules synthesized both chemically and biologically (genetically). This rich source of molecular collection can be screened for a function or affinity of interest. Genetically-encoded combinatorial libraries such as phage display libraries have taken a special place in the flourishing field of combinatorial technology particularly for the development of novel classes of tumor-avid molecules[30,31]. Genetic encoding makes it possible for molecules of a library with specific binding properties to be resynthesized and rescreened.

Construction of phage display random libraries has been one of the most fascinating and impressive developments in the area of phage display technology. In fact, phage display as a high throughput approach provides a robust basis for the production of massive libraries of molecules with different structural properties. Random peptide libraries are known to be one of the most frequent types of phage display libraries. These random libraries are made *via* cloning degenerate oligonucleotide sequences into a gene encoding one of the phage coat proteins. Each phage clone expresses a unique amino acid sequence on its surface, but the whole library may harbor billions of peptides[12,32,33]. Peptides in these random libraries have a length ranging from 5 to 20 amino acids. However, heptapeptide and dodecapeptide libraries - being commercially available - are the most broadly used peptide libraries.

These huge random libraries offer the advantage of using affinity selection to identify ligands with great specificity and affinity to any desired target. Interestingly, this procedure eliminates the need for any prior knowledge of characteristics of the target molecule. Multiple rounds of affinity selection are an extremely efficient and powerful strategy for the selection of very rare but highly specific binders from a huge pool of variants. To date, phage libraries have been applied to identify ligands against a wide variety of targets including purified proteins, antibodies, enzymes, cell surface receptors and in particular cancer-associated antigens[30,34,35]. Screening of phage libraries can be performed both in vitro against cultured cells and in vivo within the body of living animals through systemic circulation thereby leading to the isolation of cell-specific or organ-specific ligands, respectively[36-38].

Screening of a phage library over the desired target is carried out through an affinity selection-based process called biopanning. This process provides a means for selective enrichment of target-binding phages. In this approach, a library of phage-displayed peptides is passed over a plate coated with the target for which a binding ligand is sought. Bound phages are captured, while non-binders are eliminated by several washing steps. Due to the stability of phages, specifically bound phages can be eluted by acidic pH, denaturants (reducing agents) or ionic strength. Recovered phages are then amplified by infection of *E.coli* cells and undergo several - typically three to five - additional cycles of binding/amplification. This cyclic process of binding, washing, elution and amplification ultimately results in selective enrichment of high affinity binding sequences to the target. These enriched phage clones obtained from the last round are subjected to DNA sequencing in order to characterize and identify peptides with the highest affinity and specificity towards the target[39-41]. Because biopanning is a multistep selection procedure, very rare specific binders can be selected and amplified from a large background of phages with irrelevant binding property. One considerable benefit of phage display-based biopanning is that this screening modality provides various routes for the presentation of target to the library. This enables researchers to conveniently adapt screening protocol to the distinctive requirements of different targets. Target molecules can be immobilized onto a support such as coated tubes or plates, columns or magnetic beads and phage library is subsequently exposed to the immobilized target. Although being time-consuming, in this strategy only the target molecule is presented to the library. Targets can also be elements on the surface of cells either cultured in vitro or existent in different organs of the body of living animals. In this platform, target molecules are exposed in a (quasi)-natural position to the displayed library. But, the likelihood of binding of displayed peptides to many non-target molecules is an issue deserving further consideration[26,41].

Peptide ligands identified from phage libraries can be employed as leads in drug design and in vaccine development. Furthermore, some isolated peptides are bioactive and can inhibit the function of their target molecule (*e.g.,* enzyme inhibition). Therefore, these ligands represent potential to be used for various diagnostic and therapeutic applications. One of the most significant aspects of biopanning through phage libraries is searching for ligands - for example peptide ligands-specific to target molecules over-expressed on the surface of malignant cells. Peptides selected through phage libraries have been utilized for imaging, diagnosis, and treatment of a wide range of tumors such as glioblastoma, melanoma, leukemia, prostate and thyroid cancer[30,42-44]. These tumor-homing ligands are of great relevance for biomedical purposes and provide a means for selective delivery of various gene therapy vectors and chemotherapeutic drugs into neoplastic cells. Molecularly targeted delivery of anticancer agents enhances the therapeutic index and mini-

mizes the toxicity of these compounds.

BACTERIOPHAGE-BASED DIAGNOSTIC APPROACHES FOR HBV INFECTION

HBcAg, a major protein that forms the inner core of HBV virions, is regarded as one of most critical markers for detecting viral infection. The quantity of this protein demonstrates viral load as well as viral genomic DNA. HBcAg concentration in the serum correlates well with the amount of HBV genome implying its potential to be employed as a marker for viral load. Existence of anti-HBcAg antibodies in the serum can find utility as a specific serological marker for the diagnosis, monitoring of infected individuals, differentiation between acute and chronic forms of viral infection as well as epidemiological evaluations[45,46]. On the other hand, detection of antibodies generated against HBcAg is of particular importance in the identification of infected patients who are negative for HBsAg. Identification of this category of patients is crucial to impede HBV contamination of blood transfusion products[47].

Phage display has been used for surface display of HBcAg as a novel approach for the production of anti-HBcAg monoclonal antibodies. In this framework, HBcAg-displaying phage serves as an immunogen for immunization purposes and potential development of HBcAg-specific monoclonal antibodies. This bacteriophage platform also represents promise for the production of anti-HBV vaccines. Bahadir *et al*[48] applied phage display to express full-length HBcAg protein on the surface of M13 phage as fusion to minor coat protein. This recombinant phage was found to be largely immunogenic in BALB/c mice with antibody responses comparable with that of commercial HBcAg. In this work, pⅢ protein - with only three to five copies on M13 surface - was chosen as the fusion partner of HBcAg. This is due to the fact that HBcAg is approximately too large for proper display in full-length form on pⅧ protein.

Also, phages displaying surface ligands (such as peptides or antibodies) with tight binding ability to HBcAg can be used as diagnostic tools for the detection of HBV presence in biological samples. M13 bacteriophage bearing a surface peptide sequence that interacts tightly and selectively with HBcAg has been demonstrated to have the capability of detecting this viral antigen using phage-ELISA, phage-dot blot and immunoprecipitation assays[49]. This peptide was isolated *via* biopanning of a phage display cyclic peptide library and further analysis revealed that it can specifically bind to HBcAg but not to HBsAg and HBeAg (Hepatitis B e Antigen). HBeAg is the extracellular form of HBcAg[50]. In addition, this bacteriophage system represented the ability to detect HBcAg released from virions in HBV-positive serum samples. In another report, the aforementioned fusion phage (displaying a selective peptide towards HBcAg) was used to develop a TaqMan based real-time method as a diagnostic assay for HBV detection in positive serum samples[51]. This special strategy was formulated based on the idea of phage display-mediated immune-polymerase chain reaction (PD-IPCR) originally introduced by Guo *et al*[52] PD-IPCR had previously been shown to be a highly sensitive method for the detection of viruses such as Hantaan virus nucleocapsid protein. Generally speaking, detection of HBcAg in blood samples can be performed by radioimmunoassay, ELISA and enzyme immunoassay[45,46,53]. The necessity of using monoclonal or polyclonal antibodies in these assays renders the procedure of HBV detection challenging, laborious and time-consuming. Development of a bacteriophage-based diagnostic method - with the capacity of rapid propagation in bacterial cells within several hours -immensely facilitates detection of HBV infection. PD-IPCR hugely increases detection sensitivity (100 to 10, 1000 fold) compared to conventional phage-ELISA. This enormous rise in sensitivity arises from remarkable amplification power of PCR[54]. The results of this study suggest that PD-IPCR can be used as an alternative to phage-ELISA for HBcAg diagnosis.

BACTERIOPHAGE-BASED THERAPEUTIC APPROACHES FOR HBV INFECTION

Initial efforts for bacteriophage-mediated transduction of mammalian cells date back to several decades ago. At the outset, chemical transfection (by using DEAE -diethylaminoethyl - dextran and lipopolyamine) was applied to transduce cultured mammalian cells with both filamentous (single stranded DNA) and lambda (double stranded DNA) phages[55-57]. Later on, Hart *et al*[58] demonstrated that filamentous bacteriophages displaying the RGD (arginine-glycine-aspartic acid) tri-peptide sequence can be selectively targeted to and subsequently internalized by mammalian cells. Although there was not any report on the capacity of bacteriophages for gene expression in eukaryotic cells, this study suggested cellular internalization of these viral agents *via* expressing surface ligands and led to the speculation that bacteriophages can be exploited as tools for the delivery of foreign genetic material into specific mammalian cells. Larocca *et al*[59] provided the first proof of principle of bacteriophages as a targeted gene delivery vehicle. They showed that M13 filamentous bacteriophages bearing a CMV (cytomegalovirus)-controlled green fluorescent protein (GFP) gene and genetically displaying fibroblast growth factor (FGF2) can target COS-1 cells in a FGF2 receptor-mediated manner triggering the expression of the phage encoded reporter gene in mammalian cells.

Targeting of various gene carriers towards specific cells is one of the most complicated issues in gene therapy that dramatically reduces toxicity concerns. To achieve successful outcomes in gene therapy, it is necessary for the therapeutic transgene of interest to be exclusively expressed in target cells sparing normal non-targeted tissues. Larocca's pioneering study laid the groundwork for potential application of bacteriophages as a unique novel

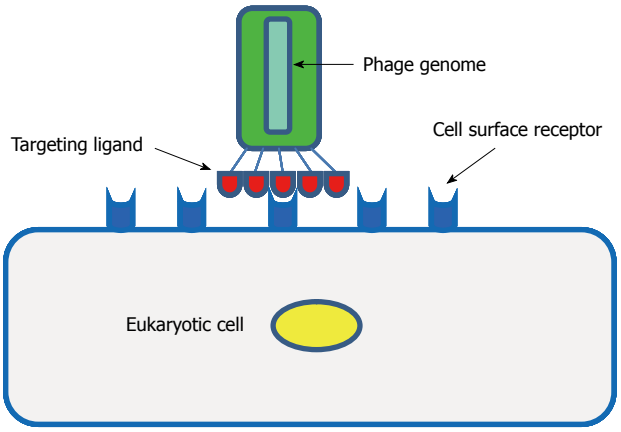

Figure 2 Cell-specific targeting of bacteriophages. Attachment of a ligand, with specific binding ability to a receptor on the surface of target cell, leads to targeted delivery of bacteriophage to the cell of interest. This ligand is fused to one of the phage coat proteins.

class of gene therapy vectors and provided strong support for the notion that bacteriophages, although having no tropism for mammalian cells, can be adapted for cell type-specific delivery of therapeutic genes into mammalian cells when properly targeted. This proper targeting can be made feasible through linking of a ligand -with binding capability to the desired cell surface receptor - to the surface of phage particles (Figure 2). Engagement of specialized cell surface receptors potentiates targeted bacteriophages to deliver their cargoes to cells through receptor-mediated endocytosis (RME)[60,61]. The use of RME potential presents a new perspective to the landscape of gene delivery. Within the past years, a variety of molecules including growth factors (basic fibroblast growth factor and epidermal growth factor), antibodies, and viral capsid proteins have been utilized for targeting bacteriophage vectors towards mammalian cells[62,63]. Phage display technology offers a powerful approach for targeted delivery of therapeutic cargoes. Bacteriophage-based surface display of peptides, poplypetides or antibodies that show preferential binding to specific receptors on the surface of target cells, organs or tissues is a highly valuable modality in the discovery of new targeting methodologies. This strategy establishes new opportunities for the design of gene delivery vectors with improved targeting properties. Combination of disease-related receptors and their corresponding ligands forms a foundation based on which new systems for targeted delivery can be developed. In this regard, natural or artificial ligands with the capability of binding to receptors distinctively expressed on the surface of target cells might be used to direct therapeutics towards sites of interest.

Hepatitis B virus makes interactions with the surface of liver cells resulting in virus binding to the cell surface. This surface binding allows subsequent internalization of viral particles into hepatocytes. Hepatitis B virus uses some surface molecules- peptides or proteins- for interacting with hepatocytes. As previously noted, PreS1 is one of the most crucial elements underlying the attachment of HBV particles to hepatocytes. One potential means for bacteriophage-based targeted delivery into liver cells - as the host cell of hepatitis B virus- is display of surface components of HBV that are necessary for binding to hepatocytes. This approach makes it possible to exploit phage display technique for the development of targeted platforms in order to treat HBV infection. Tang *et al*[64] took advantage of this strategy to develop a bacteriophage vehicle with potential application in human gene transfer and gene therapy. They fused several polypeptides of the PreS1 region of HBV in frame to the C-terminus of 10B capsid protein of phage T7. Their experiments demonstrated the efficacy of $PreS1_{60\text{-}108}$ polypeptide in transfection of HepG2 (Hepatoblastoma G2) cells. Also they found that higher phage concentrations and longer incubation periods trigger higher transfection efficiency highlighting the fact that phage internalization through displayed polypeptide of the PreS1 region occurs in a dose- and time-dependent manner. This study suggests that $PreS1_{60\text{-}108}$ region can be used as a targeting ligand to confer hepatocytic tropism to bacteriophages or any other gene and drug delivery vehicle. These targeted delivery vehicles offer a means for the delivery of therapeutic cargoes into liver cells thus providing a platform for the treatment of HBV infection, liver cancer and other hepatocyte-related disorders.

Another framework represented by phage display for the treatment of HBV infection is the utilization of random phage display libraries - both peptide and antibody libraries - in order to obtain ligands with selective binding to surface molecules of hepatitis B virus. These HBV-binding ligands might prove useful in targeted delivery of various carriers into HBV-infected haptocytes and also as antiviral drugs against HBV infection. Wang *et al*[65] screened a commercial phage display dodecapeptide library against purified PreS1 protein of HBV. This library contained peptide variants as fusion to minor coat protein (pⅢ) of M13 phage. They identified several distinct PreS1 binding peptides among which one peptide was the most enriched with the tightest ability for binding to the target protein. Further analysis revealed that this peptide strongly decreased the attachment of HBV virions to the PreS1 antibody in a dose-dependent manner exhibiting its capability in effective blocking of the relevant epitope of PreS1 protein. Peptide ligands or motifs obtained from phage display libraries with the merit of recognizing and blocking the surface molecules of HBV offer promise to be a candidate for the treatment of HBV infection. As a result, they can be utilized for the design of novel drugs against HBV. In another study, Deng *et al*[66] reported screening of a random peptide library against the PreS region of HBV as target. In contrast to the previous case, in this work peptides were displayed as fusion to major coat protein (pⅧ) of M13 phage. On the other hand, peptides of this library were structurally constrained in which random peptides were enclosed in a loop flanked by a pair of cysteine residues. Five rounds of biopanning gave rise to the enrichment and selection of PreS-binding

constrained peptide ligands.

Antibodies with the ability of being internalized into HBV-infected cells hold enormous potential for the development of treatment methods against hepatitis B infection. Park *et al*[67] constructed a large nonimmunized/naive human antibody phage library in scFv (single-chain variable fragment) format. Peripheral blood mononuclear cells (PBMC) of nonimmunized healthy donors were used as a source of lymphoid tissue. Subsequent to screening of this phage library, two functional anti-PreS1 scFvs were obtained. Survey of biological activity of the isolated scFvs established that they can recognize PreS1 and neutralize the binding of PreS1 protein and HBV virions to a human hepatoma cell line. As interaction between PreS1 (on the surface of HBV) and hepatocyte is vital for HBV infection, inhibition of this interaction *via* the identified scFvs may be promising in immunoprophylaxis and immunotherapy against HBV infection. Furthermore, this type of phage library can be an advantageous and applicable source of antibodies to any viral or malignant cell target. Wen *et al*[68] exploited screening of a natural immune antigen binding fragment (Fab) antibody phage library against HBsAg. This library had been constructed from the lymphocytes of a volunteer immunized with HBsAg. A number of Fab fragments with considerable ability for binding to the target antigen were identified following several rounds of biopanning. Subsequently, these Fab fragments were reconstructed into scFvs for further analysis. This reconstruction of display format of the isolated antibodies was due to the fact that scFvs represent interesting features including low molecular weight and convenient permeability while retaining binding capacity. All scFvs maintained a high affinity against HBsAg on the membrane of HBV-infected cells. Further analysis revealed that one of scFvs is heavily internalized into HBsAg-positive HepG2.2.15 cells. On the other hand, as transferrin is a marker for clathrin-mediated endocytosis, colocalization of this scFv with tranferrin suggests the possible role of clathrin-mediated endocytic pathway in the trafficking of scFv into infected cells.

It is interesting to note that phage display technology presents the possibility for affinity maturation of HBV-binding ligands. In this context, phage display can find utility as a tool for the production of a mutant antibody library with the intention of augmenting affinity of an antibody. Yang *et al*[69] made use of phage display library to enhance the affinity of an anti-PreS1 antibody with HBV-neutralizing activity in chimpanzees but inadequate affinity for clinical application in human. They generated HCDR3 (heavy chain complementarity determining region)-randomized library in scFv-phage display format. Biopanning of phage-displayed library against the PreS1 antigen resulted in selecting several affinity-matured scFv variants of the original anti-PreS1 antibody. These antibody mutants, with higher affinity towards target antigen, present improved potency in neutralizing HBV infection in comparison with wild type antibody thereby providing more efficacious tools for anti-HBV immunotherapy.

BACTERIOPHAGE-BASED VACCINE DEVELOPMENT FOR HBV

Many vaccines are designed based on recombinant viral and bacterial carriers. One major complication of these vaccines in translation to the clinic is their ability to generate strong immune responses that result from previous exposure of human cells to these bacterial and viral agents[70]. The use of bacteriophages as viruses whose naturally occurring hosts are non-human prokaryotic cells can bring new opportunities to the field of immunotherapy and vaccine development. Bacteriophages have recently been introduced as efficient vehicles for vaccine delivery. Bacteriophage-carried antigens have the capacity to trigger humoral and cell-mediated immune responses. Various bacteriophages including filamentous phage, lambda phage, T4 and T7 can be exploited for vaccination purposes[71].

Bacteriophages represent some advantages for the development of vaccine delivery systems. Bacteriophage-based antigen delivery platforms are low-cost and stable under relatively extreme environmental conditions. They can be readily and simply produced in large quantities because of their exponential multiplication on a simple bacterial host. These vaccine vehicles offer an approximately large cloning capacity; for example up to 20 kb for lambda-phage-based vaccines[71-73]. This provides potential for the delivery of multiple different constructs (different vaccines or multiple copies of the same vaccine) by a single phage particle, inclusion of adjuvant (cytokine and chemokine) genes on the phage vaccine, and also cloning of large intron-harboring genes of eukaryotes. The latter property is of considerable significance to tailor bacteriophage vaccines for eukaryotic parasites such as *Plasmodium falciparum* (causal pathogen of malaria) or *Trypanosoma brucei* (causal pathogen of trypanosomiasis); disease-causing agents with complicated life cycles or antigen switching activity[73]. In contrast to vaccines based on eukaryotic viruses, bacteriophages are lacking ability to propagate in eukaryotic cells that excludes the possibility of vaccine replication in human cells. With the birth of phage therapy concept in the initial decades of the 20th century, there have been a multitude of studies - particularly in Eastern Europe countries - in which these prokaryotic viruses were safely utilized for the treatment of bacterial infections[74,75]. This implies the fact that there is a long tradition of safe application of bacteriophages in human for clinical purposes.

There are two main strategies for the exploitation of bacteriophages as vaccine delivery platforms: phage DNA vaccines and phage display vaccines[76]. In phage DNA vaccines, the sequences necessary for the synthesis of vaccine antigen under the control of an appropriate eukaryotic expression cassette are inserted into the phage genome. Following DNA packaging in vitro and propagation of phage virions on the bacterial cells, the

whole recombinant phage particles are used as a delivery carrier for DNA vaccination and immunization of the host. When introduced into APCs (antigen presenting cells) of the host immune system, the phage protein coat is removed and the vaccine-encoding DNA is expressed. Compared with standard plasmid DNA vaccination, phage DNA vaccines have been indicated to mount enhanced antibody responses in mice and rabbits[10]. March *et al*[72] used lambda-phage DNA vaccine containing HBsAg (λ-HBsAg) for immunization of rabbits and mice. Compared with those vaccinated with equivalent plasmid construct encoding HBsAg, λ-HBsAg-immunized animals presented a higher titer of anti-HBsAg antibodies and this antibody response did not show any sign of reduction more than six months post-immunization. Clark *et al*[77] incorporated an expression cassette containing HBsAg of hepatitis B virus into the genome of lambda phage and used this phage-based vaccine for vaccination against HBV in rabbits. Furthermore, they compared the immunization capacity of λHBsAg with a commercially available protein vaccine, EngerixB, that is made up of HBsAg recombinant protein. They demonstrated that the phage construct in comparison with Engerix B protein vaccine is able to produce significantly higher antibody responses. This difference can be ascribed in part to the adjuvant effects of lambda phage particles. Immunostimulatory effects of bacteriophage particles have been previously shown in several studies[74,78]. In another study, Clark and March intramuscularly injected mice with λ-gt11 phages harboring HBsAg of hepatitis B virus[71]. Mice groups vaccinated with λ-HBsAg were found to yield elevated anti-HBsAg responses in comparison with unmodified λ-gt11 phage or HBsAg-containing plasmid (naked DNA). Additionally, a potent antibody response was observed against the coat protein of the carrier phage lambda. This phenomenon leads to the formation of immune complexes that play a critical role in effective targeting of antigen presenting cells thereby boosting efficiency of the delivery system. Although this finding reflects momentousness of initial priming of the immune system against the phage carrier, investigation of the impact of anti-phage response on the efficacy of the delivery system remains to be elucidated.

In phage display vaccines, bacteriophages are engineered in order to display a specific peptide or protein with antigenic property on their surface. A number of studies have revealed the potential of phage displayed antigens for the development of vaccines against pathogens such as hepatitis C virus[79], HIV-1 (Human Immunodeficiency Virus-1)[80], *Plasmodium falciparum*[81], *Neisseria meningitidis*[82], as well as diseases including Alzheimer[83] and cancer[84] in animal models. These reports have successfully exhibited the capability of these vaccines in the activation of immune responses. In this regard, a vaccine against *Taenia solium* pig cysticercosis is known as the first vaccine developed through phage display for a large animal like pig[85]. Tan *et al*[86] displayed major antigenic region of HBV, the immunodominant region (amino acids 111-156) of S-HBsAg, on the surface of T7 phage as fusion to the C-terminal end of 10B capsid protein. This region has proven to be capable of eliciting protective antibodies. T7 phage-HBsAg construct was indicated to generate desirable antigenic responses with the guinea pig anti-HBsAg antibody and human anti-HBsAg serum in ELISA tests. This observation implies the merit of this phage construct for the detection of antibodies produced against HBsAg in infected individuals. Moreover, the sera purified from rabbits immunized with T7 phage-HBsAg exhibited high levels of anti-HBsAg antibodies suggesting the potential of these phage particles as candidates for vaccine production. The results were in support of antigenic and immunogenic characteristics of the immunodominant region of HBsAg displayed on T7 phage. This antigenicity and immunogenicity represent the potential of T7 engineered phage particles to be employed as immunological reagents in detection assays for HBV infection also as immunogens for the development of economical vaccine platforms.

In the aforementioned reports, the capacity of bacteriophage-based vaccines in inducing humoral immunity was brought into focus. However, specific cellular immune responses also play substantial roles in generating anti-virus immunity thereby taking a special place in the establishment of bacteriophage-based vaccines against HBV infection. Keeping this in mind, it is of particular importance to explore whether vaccines developed based on bacteriophage particles can stimulate T cell responses especially antigen-specific cytotoxic T cell (CTL) responses. Wan *et al*[87] displayed a MHC (major histocompatibility complex) class I molecule (H-2d)-binding peptide called HBs_{28-39}, an epitope generated by exogenous processing of hepatitis B virus surface anigen, on the surface of filamentous phage particles (as fusion to the pⅧ coat protein) and injected the constructed phage into BALB/c mice without adjuvants. Several days following injection of low doses of phage particles into mice, a specific CTL response for MHC class I-restricted HBs_{28-39} epitope was found. This study suggested that filamentous phage particles can be processed by macrophages for presentation by MHC-I molecules. In another report, Manoutcharian *et al*[88] grafted a T-cell epitope that was predicted through analysis of the *Taenia crassiceps* proline-rich protective antigen KETc7 into immunoglobulin heavy-chain CDRs and displayed it on the surface of filamentous bacteriophage particles. The $CD4^+$ and $CD8^+$ T cells isolated from mice immunized with this phage display vaccine stimulated the generation of interferon gamma (INF-γ). The results indicated that phage particles can be efficiently processed and presented by MHC class I molecules eliciting strong CTL responses that yield ultimate resistance to challenge by infection. In the study conducted by Hashemi *et al*[89]recombinant filamentous phage particles containing the expression cassette of HSV-1 (Herpes Simplex Virus 1) glycoprotein D were used for vaccination of mice. Glycoprotein D is necessary for viral entry, mediates fusion of membranes following viral attachment

and is considered as an important target for the immune system[90]. After inoculation of mice with different titers of phage particles, both humoral and cell-mediated immune responses were measured in the immunized mice. In this work, antigen specific cellular response was evaluated through ELISA measurement of secreted granzyme B in the supernatant of effector-target cells co-culture. Granzyme B, a serine protease, is regarded as one of the most significant cytotoxic mediators exocytosed by CTLs upon specific recognition of antigens presented by self cell surface MHC class I molecules and triggers granule-mediated apoptosis through cleavage of different cellular substrates. The results of the study exhibited that there is a dose-response relationship in both antiviral neutralizing antibody and specific cellular immune responses. Therefore, phage virions displaying a fusion antigenic peptide can be taken up and subsequently processed by both MHC class I and class Ⅱ in antigen presenting cells and in this way stimulate the immune response to the phage-displayed epitope. In another work, it was revealed that fd virions that displayed peptide RT2 (corresponding to residues 309 to 317 of the reverse transcriptase of HIV-1) harbor the capability of priming a CTL response specific to RT2 epitope in human cell lines[91]. A T-helper epitope (pep23 corresponding to residues 249 to 263 of the reverse transcriptase of HIV-1) is required for successful priming of specific CTL response against RT2 epitope. This results in the activation of antigen specific $CD4^+$ T cells. Furthermore, HLA-A2 transgenic mice that were immunized with bacteriophages displaying RT2 peptide mounted an efficient and specific anti-HIV-RT2 CTL response.

These studies reflect the fact that bacteriophages offer potential to elicit T cell-mediated immunity in particular antigen specific cytotoxic T cell responses. The ability of bacteriophages for inducing cellular immunity, together with humoral immune response, can have significant implications for the development of bacteriophage-based vaccine platforms.

In addition to phage DNA and phage display vaccines previously mentioned, phage display methodology can also be used in another manner for vaccine development. In this approach, a specific antiserum or the serum of convalescent individuals is employed as a means for screening phage peptide libraries to recognize new protective antigens or mimotopes - peptides mimicking secondary structure and antigenic characteristics of a protective protein, carbohydrate or lipid, although bearing a different primary structure - as novel potential vaccines against a certain disease[92-94].

Another practical framework for the use of bacteriophages in vaccine design, inspired by combining phage DNA and phage display vaccines, is development of hybrid phage vaccines. This more recently established methodology, as its name represents, takes advantage of both previously mentioned approaches of phage DNA and phage display vaccines. In this strategy of vaccine development, a DNA vaccine is inserted into the phage genome, while a phage display variant of the antigen encoded by the incorporated DNA vaccine is expressed on the phage surface. This vaccine type exhibits a more considerable efficacy in targeting of both humoral and cellular components of the immune system[10,76].

Targeting of bacteriophages towards APCs is another important issue which is worthy of consideration in the development of bacteriophage-based vaccines. Surface modification of bacteriophage vaccines through specific molecules can be used as a means for preferential targeting of these vaccines towards certain types of immune cells such as APCs. With regard to targeting of bacteriophage vaccines towards cells of the immune system, dendritic cells (DCs) as the most important category of professional APCs have received considerable attention. DCs make essential contribution to the initiation of the immune response. These specialized immune cells are able to sample antigens from their surrounding environment, process the antigens that have entered the endocytic pathway, and ultimately present the processed antigens as immunostimulatory peptides to T lymphocytes in the context of MHC antigens. DCs, in addition to playing roles in priming naive T cells, are also involved in the growth and secretion of immunoglobulin molecules by B lymphocytes. As a consequence of these diverse functions, DCs support a major role in battle with viral infections and other pathogenic conditions through inducing both humoral and cellular immunity[95]. Previous studies have demonstrated the potential of DCs as effective vaccines in human clinical trials[96].

Currently, the lack of DC-specific molecules has made the design of DC-targeting strategies challenging. Great efforts have been made to develop optimal means for the delivery of immunogenic antigens to DCs. Targeting of molecules to DCs is considered a potentially helpful vaccination strategy in eliciting anti-virus immune responses. Undoubtedly, the efficacy of bacteriophage-based antigen delivery systems can be improved through targeting of bacteriophage particles to DCs. It has been demonstrated that phage peptide libraries can be exploited to identify ligands with specific binding ability to DCs. These peptides make it possible to specifically guide immunogenic antigens to DCs through molecules expressed exclusively on the surface of these immune cells. In line with this, Curiel *et al*[97] screened a PhD.12-mer peptide phage display library to obtain human DC-homing peptides. The isolated DC-binding peptides were subsequently fused to the NS3 (nonstructural protein3) of hepatitis C virus - as a model immunogen and as an antigen proposed for HCV vaccines - to facilitate DC capture and presentation. This genetic fusion also preserved the selectivity of DC targeting and antigen immunogenicity. The NS3-DC-peptide fusion was efficiently presented to $CD4^+$ and $CD8^+$ T cells obtained from HCV-positive blood cells and induced antigen specific activation and proliferation of T lymphocytes. In chimeric NOD-SCID (Nonobese Diabetic/Severe Combined Immunodeficiency) mice transplanted with human cells, NS3-DC-peptide fusion

was able to prime naive $CD4^+$ and $CD8^+$ T cells for potent antigen specific proliferation and cytokine secretion. The capacity of DC-targeting peptides isolated from phage display libraries to direct immunogenic antigens to dendritic cells may present a novel platform for vaccine development. In another report, selective targeting ability of the above mentioned DC-targeting peptide was used to deliver a therapeutic siRNA into DCs. In this study, DC-specific 12-mer peptide was fused to nona-D-arginines (9dR) to selectively direct siRNA - specific to an envelope sequence of dengue virus - to DCs[98]. Dendritic cells and macrophages are the major in vivo targets of dengue virus being regarded as the predominant infected cell types. The results of the study indicated the potential of this DC-targeting approach for targeted delivery of specific therapeutic siRNA to dendritic cells. This effectively suppressed dengue virus replication leading to simultaneous decrease of viral load and aberrant cytokine responses in DCs. In another study, a semi-synthetic scFv antibody phage display library in combination with fluorescence-activated cell sorting was employed to isolate antibodies that bind to subpopulations of DCs present in human peripheral blood[99]. These DC-targeting scFv antibody fragments can be genetically fused to antigens or chemically coupled to nucleic acids thereby providing an immunotherapeutic means for targeted delivery of vaccines to DCs.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (10): Alcoholic Liver Disease

Diagnosis of alcoholic liver disease

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Abstract

Alcohol is a hepatotoxin that is commonly consumed worldwide and is associated with a spectrum of liver injury including simple steatosis or fatty liver, alcoholic hepatitis, fibrosis, and cirrhosis. Alcoholic liver disease (ALD) is a general term used to refer to this spectrum of alcohol-related liver injuries. Excessive or harmful alcohol use is ranked as one of the top five risk factors for death and disability globally and results in 2.5 million deaths and 69.4 million annual disability adjusted life years. All patients who present with clinical features of hepatitis or chronic liver disease or who have elevated serum elevated transaminase levels should be screened for an alcohol use disorder. The diagnosis of ALD can generally be made based on history, clinical and laboratory findings. However, the diagnosis of ALD can be clinically challenging as there is no single diagnostic test that confirms the diagnosis and patients may not be forthcoming about their degree of alcohol consumption. In addition, clinical findings may be absent or minimal in early ALD characterized by hepatic steatosis. Typical laboratory findings in ALD include transaminase levels with aspartate aminotransferase greater than alanine aminotransferase as well as increased mean corpuscular volume, gamma-glutamyltranspeptidase, and IgA to IgG ratio. In unclear cases, the diagnosis can be supported by imaging and liver biopsy. The histological features of ALD can ultimately define the diagnosis according to the typical presence and distribution of hepatic steatosis, inflammation, and Mallory-Denk bodies. Because of the potential reversible nature of ALD with sobriety, regular screening of the general population and early diagnosis are essential.

Key words: Alcoholic liver disease; Diagnosis; Alcohol screening; Histology; Mallory-Denk bodies; Prognosis

Core tip: The diagnosis of alcoholic liver disease (ALD) can be challenging and in most cases, the diagnosis will be established by thorough history, clinical and laboratory findings. However, in uncertain situations, it can be supported by imaging and liver biopsy results. Histological features of ALD can ultimately define the diagnosis according to the typical presence and distribution of hepatic steatosis, inflammation, and Mallory-Denk bodies. Clinical and laboratory parameters can help with establishing the prognosis of ALD in more advanced and severe cases and with determining the therapeutic approach.

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INTRODUCTION

Alcohol is a hepatotoxin that is commonly consumed worldwide and is associated with a spectrum of liver injury including simple steatosis or fatty liver, alcoholic hepatitis, fibrosis, and cirrhosis. Alcoholic liver disease

(ALD) is a general term used to refer to this spectrum of alcohol-related liver injuries[1,2].

Excessive alcohol consumption is a risk factor for a multitude of adverse health consequences and is indeed one of the leading causes of preventable morbidity and mortality worldwide[3] with a significant burden attributable to ALD[4,5]. Excessive or harmful alcohol use is ranked as one of the top five risk factors for death and disability globally[6] and results in 2.5 million deaths and 69.4 million annual disability adjusted life years[7]. In the United States, almost 9% of adults meet criteria for an alcohol-use disorder[8] with alcohol use disorders ranking in the top 20 leading diseases contributing to disability adjusted life years[9] and resulting in approximately $223.5 billion of societal costs annually[10].

There is a strong correlation between the prevalence of ALD, specifically cirrhosis, and a country's annual per capita alcohol consumption. Levels of alcohol consumption vary geographically with Eastern European countries having the highest annual per capita consumption (15.7 L per person), while North Africa and the Middle East have the lowest annual per capita consumption (1.0 L per person)[11]. In the United States, the estimated annual per capita consumption of alcohol is 8.4 L per person[12].

Rates of ALD are highest in countries with the highest rates of alcohol consumption including Eastern Europe, Southern Europe and the United Kingdom. In 2010, ALD resulted in 493300 deaths worldwide and 14.5 million disability adjusted life years with alcoholic cirrhosis comprising 47.9% of all liver cirrhosis deaths[11]. In the United States, 31522 adults died from liver cirrhosis in 2009, with 48.2% of these deaths attributable to alcohol[13].

While alcohol is a well established hepatotoxin with higher levels of consumption associated with increased risk of development of ALD, no absolute threshold of alcohol consumption is necessary for the development of liver injury, and no direct linear correlation between level of alcohol consumption and severity of ALD has been established.

Approximately 60%-90% of individuals who drink more than 60 g of alcohol per day have been shown to have hepatic steatosis[14,15]. However, less than half of individuals with alcoholic steatosis, who continue to drink alcohol, will progress to fibrosis and only 10%-20% will eventually progress to cirrhosis[16,17]. Nonetheless, once steatohepatitis has developed, the risk of development of cirrhosis is increased compared with simple steatosis[18]. In addition, individuals who have demonstrated steatohepatitis who continue to drink alcohol or who develop symptomatic alcoholic hepatitis have higher rates of progression to cirrhosis compared with those who subsequently abstain from alcohol consumption or who have never had an episode of symptomatic alcoholic hepatitis. Alcoholic cirrhotics who abstain from alcohol consumption for at least 1.5 years have improved survival rates compared to those that continue to drink[19].

The underlying mechanisms which make some individuals more susceptible to severe forms of ALD are not entirely well understood and are likely multifactorial. Several risk factors have been identified that appear to be correlated with development and progression of ALD including amount and pattern of alcohol consumption, gender, ethnicity, age, obesity, co-existing chronic viral hepatitis, iron overload, smoking, and host genetic factors[20-27].

GENERAL DIAGNOSTIC APPROACH TO ALD

The diagnosis of ALD can generally be made based on clinical and laboratory features alone in patients with a history of significant alcohol consumption after other etiologies for chronic liver disease have been ruled out. However, the diagnosis of ALD can be clinically challenging as there is no single laboratory or imaging study that can confirm the diagnosis. Furthermore, patients may be completely asymptomatic, have no clinical signs of early ALD or early cirrhosis and may have normal liver enzymes. In addition, patients may have co-existing risk factors for non-alcoholic fatty liver disease such as obesity and diabetes and some may not be entirely forthcoming as to their degree of alcohol consumption.

In general, ALD should be suspected in patients with a significant history of alcohol use who present with abnormal serum transaminases, particularly if the level of aspartate aminotransferase (AST) is greater than that of alanine aminotransferase (ALT), hepatomegaly, clinical signs of chronic liver disease, radiographic evidence of hepatic steatosis or fibrosis/cirrhosis, or who have had a liver biopsy showing macrovesicular steatosis or cirrhosis.

Patients with ALD may or may not have elevated serum aminotransferase levels. The absolute level of liver enzyme elevation does not correlate well with the severity of ALD, however, the pattern of elevation in transaminases is helpful in making a diagnosis of liver injury due to alcohol as AST is typically two to three times greater than ALT in alcoholic liver injury[28]. They will also typically have an elevated serum gamma-glutamyltranspeptidase (GGT)[29]. However, it is important to rule out other etiologies for the patient's liver disease before making a definitive diagnosis of ALD, including chronic viral hepatitis, autoimmune hepatitis, hemochromatosis and drug related hepatotoxicity. In some cases, when the diagnosis is unclear, a liver biopsy may be warranted.

SCREENING FOR ALCOHOL USE DISORDERS

To review, one standard alcoholic drink is considered any alcoholic beverage that contains 14 g of alcohol. Examples of a standard drink include 12 ounces of regular beer, 8-9 ounces of malt liquor, 5 ounces of wine and 1.5 ounces of distilled spirits. Men who consume more than 4 standard drinks in any single day (or more than 14 drinks per week) and women who consume more than 3

Table 1 Typical clinical features of alcoholic liver disease[36-40]

Spectrum of ALD	Clinical presentation
Alcoholic fatty liver	Asymptomatic
Alcoholic hepatitis	Jaundice
	Anorexia
	Fever
	+/- RUQ/epigastric pain
	+/- Abdominal distention due to ascites
	+/- Proximal muscle weakness
	+/- Confusion due to HE
Compensated cirrhosis	Asymptomatic
	Anorexia
	Weight loss
	Weakness
	Fatigue
	Muscle cramps
	Amenorrhea or irregular menses
	Impotence, infertility, loss of sexual drive
Decompensated cirrhosis	Jaundice
	Pruritus
	GI bleeding
	Weight gain
	Abdominal distention due to ascites
	Lower extremity edema
	Easy bruising
	Sleep disturbances
	Confusion

ALD: Alcoholic liver disease; GI: Gastrointestinal; RUQ: Right upper quadrant; HE: Hepatic encephalopathy.

in any single day (or more than 7 drinks per week) are at increased risk for alcohol-related problems[30].

Worldwide, approximately 20%-30% of patients who present in primary care settings engage in hazardous or harmful drinking[31]. Hazardous drinking is defined as a pattern of drinking that increases the risk of physical or psychological problems[32] and harmful drinking is defined as a pattern of drinking that results in such problems[33]. Persistent drinking despite adverse health or psychological consequences constitutes an alcohol-use disorder which includes a spectrum of disease ranging from alcohol abuse to alcohol dependence[34]. At the severe end of the spectrum, individuals who are alcohol dependent suffer from a brain disorder characterized by loss of control over their drinking, alcohol craving, frequent drinking, continued drinking despite negative consequences, tolerance, withdrawal and disability[35]. It is recommended that health care providers screen for and counsel risk drinkers as part of routine medical and preventive care[35].

All patients who present with clinical features of hepatitis or chronic liver disease (Table 1) or who have elevated serum transaminase levels should be screened for an alcohol use disorder. Denial of alcohol abuse and underreporting of alcohol intake are common among alcoholics[41] and thus, clinicians should have a low threshold to screen their patients for alcohol abuse. In the United States, routine alcoholism screening is widely recommended and is now re-imbursed on an annual basis by Medicare[42]. The US Preventive Services Task Force (USPSTF) recommends routine screening of all adult primary care patients followed by a brief counseling intervention of persons who engage in risky or hazardous drinking (grade B recommendation: high certainty that the net benefits is moderate or there is moderate certainty that the net benefit is moderate to substantial)[43] and the National Institute on Alcohol Abuse and Alcoholism recommends annual screening of all adults with the use of a validated self-reporting tool[44].

Several validated screening tools that can easily be administered during a clinical visit are available to identify patients at risk for alcohol abuse. The USPSTF prefers the use of alcohol use disorders identification test (AUDIT), AUDIT-consumption (AUDIT-C) and single question screening in the primary care setting. Of the available screening instruments, the AUDIT is the most widely studied for detecting alcohol use disorders in the primary care setting[45]. The AUDIT comprises ten questions with a specific scoring system (Table 2) and requires approximately 2 to 5 min to administer. An optimal score for detecting unhealthy alcohol use in men is 5 for men (sensitivity 77%, specificity 76%) and 3 for women (sensitivity 86%, specificity 74%). A score of 6 or more for men (sensitivity 84%, specificity 76%) and 4 or more for women (sensitivity 88%, specificity 76%) is highly suggestive of alcohol dependence[47].

The AUDIT-C questionnaire, an abbreviated version of the AUDIT performs as well as the full 10 item AUDIT, and significantly better than self-reported risky drinking or the CAGE questionnaire[48]. The CAGE questionnaire, the name of which is an acronym of its four questions, is considered positive if a patient answers yes to two or more of the following questions: (1) Have you ever felt you needed to cut down on your drinking? (2) Have people annoyed you by criticizing your drinking? (3) Have you ever felt guilty about drinking? or (4) Have you ever felt you needed a drink first thing in the morning (eye-opener) to steady your nerves or to get rid of a hangover[49]?

The AUDIT-C is comprised of three questions with a specific scoring system (Table 3) ranging from 0 to 12 and takes approximately 1 to 2 min to complete. A positive screening result is a score of 3 or more for women and 4 or more for men. A score of 7 to 10 has been associated with increased risk of alcohol dependence[50]. The AUDIT-C screening tool has been shown to be 73% sensitive and 91% specific for an alcohol-use disorder and 85% sensitive, 89% specific for alcohol dependence[51]. The AUDIT-C score also serves as an excellent marker of alcohol misuse severity[52]. A positive result should prompt a more in-depth assessment of the patient's alcohol use pattern and formal evaluation for an alcohol-use disorder.

A second validated brief screening tool available with adequate sensitivity and specificity involves a single question asked of patients: "How many times in the past year have you had five (four for women) or more drinks in a day?" If a patient responds to this as one or more times, this is a positive screening result. This question has been demonstrated to be 82% sensitive and 79% specific for unhealthy use of alcohol[53].

Table 2 Alcohol use disorders identification test[46]

1 How often do you have on a drink containing alcohol?
(0) Never (skip to questions 9-10)
(1) Monthly or less
(2) 2 to 4 times a month
(3) 2 to 3 times a week
(4) 4 or more times a week
2 How many drinks containing alcohol do you have on a typical day when you are drinking?
(0) 1 or 2
(1) 3 or 4
(2) 5 or 6
(3) 7, 8, or 9
(4) 10 or more
3 How often do you have six or more drinks on one occasion?
(0) Never
(1) Less than monthly
(2) Monthly
(3) Weekly
(4) Daily or almost daily
4 How often during the last year have you found that you were not able to stop drinking once you had started?
(0) Never
(1) Less than monthly
(2) Monthly
(3) Weekly
(4) Daily or almost daily
5 How often during the last year have you failed to do what was normally expected from you because of drinking?
(0) Never
(1) Less than monthly
(2) Monthly
(3) Weekly
(4) Daily or almost daily
6 How often during the last year have you needed a first drink in the morning to get yourself going after a heavy drinking session?
(0) Never
(1) Less than monthly
(2) Monthly
(3) Weekly
(4) Daily or almost daily
7 How often during the last year have you had a feeling of guilt or remorse after drinking?
(0) Never
(1) Less than monthly
(2) Monthly
(3) Weekly
(4) Daily or almost daily
8 How often during the last year have you been unable to remember what happened the night before because you had been drinking?
(0) Never
(1) Less than monthly
(2) Monthly
(3) Weekly
(4) Daily or almost daily
9 Have you or someone else been injured as a result of your drinking?
(0) No
(2) Yes, but not in the last year
(4) Yes, during the last year
10 Has a relative or friend or a doctor or another health worker been concerned about your drinking or suggested you cut down?
(0) No
(2) Yes, but not in the last year
(4) Yes, during the last year

Skip to questions 9 and 10 if total score for questions 2 and 3 = 0.

The last brief screening tool available is a set of three questions that evaluates the typical quantity of drinks consumed on one occasion, frequency of drinking per week and maximum number of alcoholic beverages consumed on any given occasion in the past month (Table 3). A positive screen result is greater than 14 drinks consumed per week or more than 4 drinks consumed on one occasion for men and greater than 7 drinks consumed per week or more than three drinks consumed on one occasion for women or persons older than age 65. This tool has been shown to be 83% sensitive and 84% specific for alcohol abuse or dependence in the past year[54].

Validated self-report questionnaires have been shown to have both greater sensitivity and specificity for detecting alcohol abuse than blood tests for biochemical markers[55]. No single reliable diagnostic biomarker has been identified which has adequate sensitivity and specificity to be useful for general screening of alcohol consumption or abuse[56,57]. Nevertheless, biochemical markers may play a role in alcohol abuse screening when the clinician suspects heavy drinking in a patient who denies it (see laboratory studies section).

PHYSICAL EXAMINATION

A detailed physical examination should be performed to evaluate the patient for evidence of chronic liver disease (Table 4). The physical examination findings in patients with ALD will vary depending on the severity of disease and range from a completely normal examination to physical signs of cirrhosis with severe decompensation (Table 4). Physical findings may be normal and non-diagnostic particularly in patients with mild ALD, steatosis or early cirrhosis. Patients with cirrhosis and portal hypertension may exhibit stigmata of chronic liver disease and if concomitant hepatic decompensation exists, may also exhibit ascites, peripheral edema, asterixis and/or mental confusion. Patients with alcoholic hepatitis will have scleral icterus and jaundice as well as tender hepatomegaly with or without ascites and if their hepatitis is severe will have asterixis and exhibit mental confusion on examination. In addition, patients with ALD typically have co-morbidities due to the concomitant toxic effects of alcohol on other organ systems and may have signs of peripheral neuropathy, muscle wasting and heart failure[68,69].

LABORATORY STUDIES

While no single laboratory test will confirm the diagnosis of ALD, common laboratory abnormalities in alcoholics have been identified and certain biomarkers are highly suggestive or indicative of ALD. Additional laboratory testing can aid in the identification of hepatic inflammation, portal hypertension, assess hepatic synthetic function and potentially aid in identifying chronic alcohol abuse.

As part of initial testing, all patients being evaluated for ALD should have a complete blood count, hepatic panel (transaminases, bilirubin, alkaline phosphatase, al-

Table 3 Brief screening tests for alcohol use disorders[44,48]

Test	Questions	Scoring	Positive result
AUDIT-C	Q1: How often did you have a drink containing alcohol in the past year?		For women ⩾ 3 points; for men ⩾ 4 points
	Never	0 points	
	Monthly or less	1 point	
	Two to four times a month	2 points	
	Two to three times per week	3 points	
	Four or more times a week	4 points	
	Q2: How many drinks did you have on a typical day when you were drinking in the past year?		
	One or two	0 points	
	Three or four	1 point	
	Five or six	2 points	
	Seven to nine	3 points	
	Ten or more	4 points	
	Q3: How often did you have six or more drinks on one occasion in the past year?		
	Never	0 points	
	Less than monthly	1 point	
	Monthly	2 points	
	Weekly	3 points	
	Daily or almost daily	4 points	
Single question screening test from NIAAA	How many times in the past year have you had five (four for women) or more drinks in a day?	One point per time	⩾ 1 time
Three question screening test from NIAA	Q1: On average, how many days per week do you drink alcohol? Q2: On a typical day when you drink, how many drinks do you have? Q3: What is the maximum number of drinks you had on any given occasion during the past month		For men, > 14 drinks per week or > 4 drinks per occasion; for women or person older than 65 years, > 7 drinks per week or > 3 drinks per occasion

AUDIT-C: Alcohol use disorders identification test-consumption; NIAAA: National Institute on Alcohol Abuse and Alcoholism.

bumin), gamma-glutamyl transferase, and an international normalized ratio (INR) checked.

If a patient has evidence of hepatocellular injury as indicated by elevated serum transaminase levels, he or she should be screened for chronic viral hepatitis with measurements of hepatitis B surface antigen, hepatitis B core IgG and hepatitis C antibody; autoimmune hepatitis with anti-nuclear antibody, anti-smooth muscle antibody and IgG4 or gamma-globulin levels; hemochromatosis with serum ferritin, serum iron and transferrin with percent iron saturation; alpha one anti-trypsin deficiency with alpha one anti-trypsin level; and serum ceruloplasmin levels and 24 urinary copper for Wilson's disease.

Common hematological findings in patients with ALD include thrombocytopenia, macrocytic anemia, lymphopenia, elevated erythrocyte sedimentation rate and an elevated INR[70,71]. Macrocytosis suggests chronic disease and may be secondary to toxicity of alcohol on bone marrow, folate or vitamin B12 deficiency, or increased lipid deposition in erythrocyte membranes. Thrombocytopenia is present in about a third of alcoholics admitted to hospitals and with abstinence will tend to normalize within 1-3 wk[72]. High density lipoprotein cholesterol, serum ferritin, and urate levels also increase as a consequence of alcohol consumption[73-76]. In addition to an elevated INR, patients with poor hepatic synthetic function will also have low serum albumin levels. Interestingly, patients who engage in chronic alcohol consumption but who do not have underlying ALD may have an elevated serum albumin level possibly secondary to effects of acetaldehyde[77].

Patients with ALD frequently demonstrate evidence of iron overload as reflected by elevated serum iron indices (ferritin and transferrin saturation) and hepatic iron concentration[75,78]. Nearly 30% of patients with ALD have increased hepatic iron stores[79] and serum transferrin saturation may approach or even exceed 60% in some cases[80]. The etiology of iron accumulation in alcoholics is unknown but may be due to alcohol suppression of liver transferrin synthesis or deregulation of hepcidin synthesis in the liver[81]. Regardless of the etiology, iron overload in ALD may be difficult to differentiate from hereditary hemochromatosis, and in fact, prior to the widespread availability of HH genetic testing, often led to misdiagnosis. In cases of significantly elevated ferritin or transferrin levels, additional testing, including a DNA analysis for HFE gene mutations, is warranted to rule out hereditary hemochromatosis.

The biochemical markers for chronic alcohol consumption that have been most commonly studied are serum GGT, AST, ALT, mean corpuscular volume (MCV) and carbohydrate-deficient transferrin (CDT)[82-84]. An AST to ALT ratio over 2 is highly suggestive of ALD[85,86]. Most patients with non-ALD have AST to ALT ratios below one. Specific IgA antibodies directed towards acetaldehyde-derived protein modifications are

Table 4 Physical findings in alcoholic liver disease[58-67]

Spectrum of ALD	Physical examination findings
Fatty liver	Normal examination
	+/- Hepatomegaly
Alcoholic hepatitis	Jaundice
	Tender hepatomegaly
	+/- Ascites
	+/- Hepatic bruit
	Proximal muscle wasting
	Decreased grip strength
	+/- Hepatic encephalopathy
	(confusion, asterixis, hippus)
Cirrhosis	Spider angiomata (face, trunk, upper extremities)
	Parotid gland enlargement
	+/- Fetor hepaticus
	Gynecomastia
	+/- Hepatomegaly
	Firm liver edge with nodular contour
	+/- Splenomegaly
	Caput medusa (abdominal wall collaterals)
	Cruveilhier-Baumgarten murmur
	Testicular atrophy
	Palmar erythema
	Digital clubbing
	Muehrcke nails (paired horizontal white bands)
	Terry nails (large white proximal nail bed)
	Hypertrophic osteoarthropathy
	Dupuytren's contracture
Decompensated cirrhosis	Cirrhotic physical finding plus:
	Jaundice
	Ascites
	Peripheral edema
	Hepatic encephalopathy
	(confusion, asterixis, hippus)

ALD: Alcoholic liver disease.

frequently seen alcoholics and thus IgA levels are increased in chronic ALD. An increased ratio of IgA to IgG is highly suggestive of ALD[87-89].

Chronic alcohol consumption is known to induce a rise in serum GGT and is a widely used index for excessive alcohol use[90,91]. However, elevated GGT alone has both low sensitivity and specificity for alcohol abuse[92,93]. GGT is not specific to alcoholism and is increased in many conditions such as obesity, advanced age, moderate alcohol consumption, all forms of liver disease including fatty liver and in particular intra and extrahepatic biliary obstruction, hepatocellular carcinoma and phenytoin use[94-97]. The sensitivity of GGT as a marker for alcohol consumption in young adults has been showed to be particularly poor even in cases of documented alcohol dependence[98].

Transferrins which have a low degree of bond with carbohydrates are collectively called CDT and are increased in the serum of alcoholics[99]. However, the mechanism in which the presence of ethanol *in vivo* causes this alteration in transferrin is largely unknown. CDT is a more sensitive marker of chronic alcohol consumption in men than women who may express higher levels of CDT under natural conditions and produce less CDT in response to heavy drinking[100,101]. In addition, some studies have shown elevated CDT levels in cirrhotic patients regardless of their alcohol consumption[102] while other studies have shown normal CDT levels in patients with chronic liver disease who abstain from alcohol[103].

No single biomarker has both adequate sensitivity and specificity for detecting chronic alcohol abuse. However, when certain biomarkers are combined, they may provide improved diagnostic yield[104]. For example, while CDT has the highest specificity for harmful or heavy alcohol consumption, combining this biomarker with GGT and/or MCV, improves sensitivity significantly (Table 5). In addition, combining CDT testing with screening questionnaires, particularly for patients in which alcohol abuse is strongly suspected but who have a negative screening questionnaire result, has also been shown to be cost effective[108].

Ethyl glucuronide (EtG), ethyl sulfate (EtS) and phosphatidylethanol (PEth) have been used with increasing frequency in the past decade to monitor abstinence from alcohol in outpatient and treatment settings[109,110]. In a study on forty patients, PEth was compared with CDT as a biomarker for active alcohol consumption and was found to be positive twice as often as CDT in patients who relapsed from abstinence while in a voluntary outpatient treatment program[111]. However, considerable inter-individual variability in PEth levels have been observed in clinical studies which may create problems with the interpretation of results and may limit the usefulness of PEth to identification of relapse from abstinence[112,113]. The utility of urinary EtG and EtS, similar to measurement of blood alcohol level, is limited to detecting recent intake of even small amounts of alcohol.

Patients with alcoholic hepatitis will typically have moderately elevated aminotransferases (less than 500 IU/mL), an AST:ALT ratio of two or greater and elevated serum bilirubin (greater than 5 mg/dL)[114,115]. Patients with severe alcoholic hepatitis may also have a leukocytosis and elevated C-reactive protein indicative of acute liver injury or concomitant infection[116].

While there are no ideal non-invasive biomarkers currently available to differentiate between simple steatosis and alcoholic steatohepatitis, newly discovered biomarkers for non-alcoholic steatohepatitis (NASH) may be potentially applied to ALD in the future. For example, serum cytokeratin-18, a marker of hepatocyte apoptosis, is a promising and accurate non-invasive test for the diagnosis of NASH [area under the receiver operating curve (AUROC): 0.83-0.91][117,118] particularly when used in combination with fibroblast growth factor-21[119]. However, additional research of the utility and accuracy of these biomarkers for use in the setting of alcoholic steatohepatitis (ASH) is necessary.

IMAGING

Current widely available imaging modalities for the liver include ultrasonography (US), computed tomography scan (CT) and magnetic resonance imaging (MRI). While

Table 5 Sensitivity and specificity of biomarkers in detecting harmful or heavy alcohol consumption[105-107]

Biomarker	AST	ALT	MCV	CDT	CDT + GGT	CDT + GGT + MCV
Sensitivity	47%-68%	32%-50%	45%-48%	63%-84%	83%-90%	88%
Specificity	80%-95%	87%-92%	52%-94%	92%-98%	95%-98%	95%

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; MCV: Mean corpuscular volume; CDT: Carbohydrate-deficient transferring; GGT: Gamma-glutamyltranspeptidase.

each of these imaging studies are useful for determining the presence of underlying liver disease, they cannot confirm alcohol use as the etiology of a patient's liver disease. Nonetheless, imaging studies can be useful for excluding other causes of abnormal liver tests in patients who abuse alcohol such as infiltrative disease, obstructive biliary pathology and neoplastic diseases of the liver[120]. Imaging can also aid in the diagnosis of cirrhosis and can be used to screen for and identify hepatocellular carcinoma.

US is a non-invasive technique that is routinely used in the initial evaluation of liver. The appearance of fat in the liver is highly variable on US, however, in general, a fatty liver will have a hyperechoic texture and macroscopic fat will appear as hyperechoic masses[121]. The sensitivity and specificity of a hyperechoic pattern on ultrasound for hepatic steatosis in patients with a liver replaced by at least thirty percent steatosis is 91% and 93% respectively. In patients who have less than thirty percent hepatic steatosis, the sensitivity is only approximately 64%[122].

Hepatic steatosis is more easily detected by a non-contrast CT scan which can be a particularly useful technique to detect macroscopic fat in the liver[123]. Measurement of attenuation differences between the liver and spleen is used to identify a fatty liver. A liver-to-spleen attenuation ratio greater than 10 hounsfield units is highly predictive of hepatic steatosis[124] and the liver attenuation index has been shown to closely predict the degree of hepatic steatosis in patients with living related liver transplantation[125]. MRI techniques in which water and fat are imaged in and out of phase may be the most sensitive and specific imaging modality for detecting hepatic steatosis (95% sensitivity, 98% specificity)[126]. However, in patients with hepatic iron overload, opposed phase MRI imaging may not be able to detect the presence of fat in the liver and MR spectroscopy may be a more useful imaging modality in these patients. As with CT imaging, MRI imaging can be prohibitively expensive as an initial study and may not provide additional diagnostic yield when compared to ultrasound in the setting of macroscopic steatosis. A newer imaging modality is currently under investigation that is controlled attenuation parameter used with transient elastography which shows promising performance for detection and quantification of steatosis but which is still not widely available[127,128].

On US, patients with fibrosis may have a coarsened echo pattern to their liver and patients with cirrhosis may have a nodular liver contour. The sensitivity of US for significant fibrosis is about 57% and 71% for patients with established cirrhosis. Overall, specificity is approximately 88%[129]. CT findings in patients with cirrhosis may include atrophy of the right lobe of the liver, hypertrophy of the caudate lobe, hypertrophy of the lateral segment of the left lobe, parenchymal nodularity, attenuation of hepatic vasculature, splenomegaly, venous collaterals and ascites[130]. Imaging features on ultrasound and MRI that may be suggestive of alcoholic cirrhosis include an enlarged caudate lobe, visualization of the right posterior hepatic notch and smaller size regenerative nodules[131,132].

Improved imaging modalities have been developed over the past decade in order to detect and quantify hepatic fibrosis and cirrhosis. These include transient elastography (FibroScan), acoustic radiation force impulse and magnetic resonance elastography. These imaging techniques measure liver "stiffness" by utilizing a transducer to transmit and measure vibration (elastic shear wave) as it propagates through the liver. The velocity of this wave as it passes through the liver correlates directly with tissue stiffness. These non-invasive radiologic studies may replace the more invasive liver biopsy in the future for accurate staging of hepatic fibrosis[133,134]. To our knowledge, however, the sensitivity and specificity of these new imaging modalities for diagnosing fibrosis and cirrhosis in patients with ALD have not yet been fully evaluated.

ROLE OF LIVER BIOPSY

A liver biopsy is not necessary for the diagnosis of ALD in most patients. Clinical findings in patients with chronically elevated characteristic liver enzymes together with a history of significant alcohol use have been found to be 91% sensitive and 97% specific for the diagnosis of ALD when compared to liver biopsy[135]. However, a liver biopsy may be useful for establishing the diagnosis in some patients if the diagnosis of ALD is not clear according to clinical presentation and laboratory studies and in patients in whom the clinician suspects more than one type of underlying liver disease. Approximately 20% of patients with a history of chronic alcohol abuse have a secondary or co-existing etiology for their liver disease[136]. A biopsy can also be useful in establishing the stage and severity of liver disease. A recent study of patients with acute deterioration of alcoholic cirrhosis suggests that early transjugular liver biopsy in these patients can also provide important diagnostic and prognostic information for the identification and treatment of a subset of patients with superimposed alcoholic steatohepatitis which can be dif-

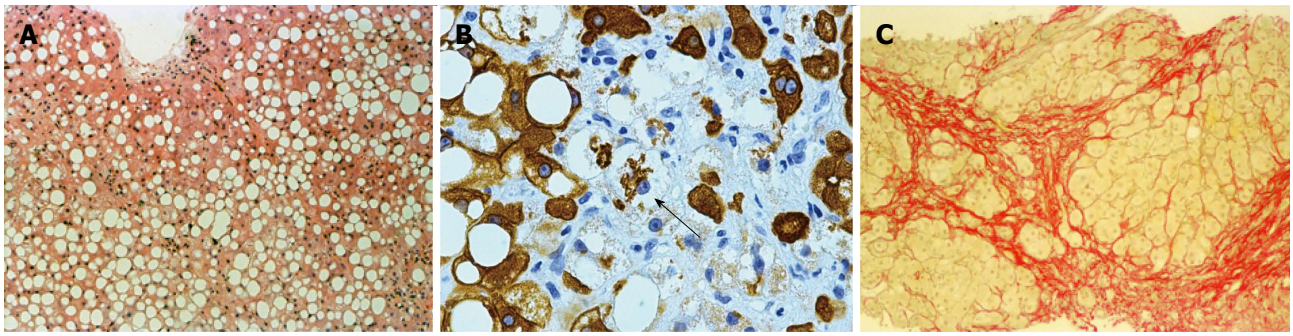

Figure 1 Histology of alcoholic fatty liver. A: Macrovesicular steatosis in alcoholic fatty liver (HE stain, × 218); B: Ballooned hepatocyte (arrow) containing a Mallory Denk body in alcoholic hepatitis (CAM5.2 stain for cytokeratins 8 and 18, × 218); C: Collagen surrounds nodules of hepatocytes in alcoholic cirrhosis (Serius red stain, × 872).

ficult to differentiate from decompensated cirrhosis on the basis of clinical and laboratory evaluation alone[137].

Currently, liver biopsy is the gold standard for the diagnosis and assessment of severity of hepatic steatosis, staging of fibrosis and is the only modality available to differentiate between bland steatosis and steatohepatitis. Liver biopsy can facilitate the differentiation between simple steatosis and steatohepatitis based on distinct histological features as described in the next section. This differentiation is of clinical significance in that it provides important prognostic information for the patient. Clinical experience with large numbers of ALD patients has demonstrated that it can be difficult to clinically predict the stage of liver disease before the development of decompensated cirrhosis[138].

Nevertheless, the liver biopsy does have limitations. It is an invasive procedure to which patients may be adverse, can cause complications, is prone to sampling error and a firm etiology for underlying liver disease may not be achieved based on histology[139]. If no treatment of ALD is being considered other than alcohol abstinence and adequate nutrition, then a histologic diagnosis is usually not warranted. Furthermore, the role of liver biopsy in making the diagnosis of alcoholic hepatitis is controversial as it carries significant risk of bleeding in the setting of coagulopathy and thrombocytopenia when using a standard percutaneous approach.

HISTOLOGY OF ALD

The histologic features of ALD on liver biopsy vary based on the extent and stage of hepatic injury. Steatosis is the most common and earliest manifestation of ALD. Steatosis in ALD is typically macrovesicular in nature (Figure 1A) in which large lipid droplets occupy nearly the entire cytoplasm of hepatocytes and displace the nucleus and other organelles peripherally[140]. The pattern for macrovesicular steatosis in ALD is typically centrilobular but it can progress to include the entire lobule in severe cases. Other early changes seen in ALD include proliferation of smooth endoplasmic reticulum, distortion of mitochondria[141] and, if severe, can be associated with giant mitochondria[142]. Giant mitochondria have been associated with all types of ALD from fatty liver to cirrhosis, and although they are not specific to ALD, the presence of giant mitochondria favors alcohol-related disease and is a good indicator of recent heaving drinking[143,144].

Steatosis may progress to steatohepatitis (ASH). ASH is characterized by liver cell damage, inflammation, and fibrosis. The typical histologic characteristics of ASH include centrilobular accentuated steatosis, hepatocyte ballooning eventually associated with Mallory-Denk bodies (MDB) (Figure 1B), a mixed inflammatory reaction of neutrophilic, lymphocytic and mononuclear cells, hepatocyte necrosis and perivenular fibrosis which can progress to spider-like pericellular fibrosis[145,146]. MDB are cytoplasmic accumulations of hepatocytic keratin intermediate filaments and are characteristic features of ASH and NASH[147,148]. In general, however, MDB are less prominent and more difficult to identify without immunohistochemistry in the non-alcoholic variant[149]. MDB can also be found in patients with amiodarone toxicity, primary biliary cirrhosis, chronic cholestasis syndromes, idiopathic copper toxicosis, Wilson's disease, Indian childhood cirrhosis, alpha-1 antitrypsin deficiency, and hepatocellular carcinoma[150-152].

Steatosis and ASH are present in approximately one third of patients with alcoholic cirrhosis and their presence usually indicates persistent alcohol abuse. Histologically, a cirrhotic liver will have fibrous septae made of collagen surrounding hepatocytes resulting in pseudolobule formation (Figure 1C) which produces a nodular appearance to the liver and which may progress from micronodular to macronodular cirrhosis over time[153,154]. Bile duct proliferation may also be prominent in the cirrhotic stage of ALD[155].

ASSESSMENT OF PROGNOSIS

Several demographic, clinical, laboratory and histologic findings can provide prognostic information for patients diagnosed with ALD. While not all patients that drink heavily will develop ALD, continued alcohol use often leads to progressive liver disease once clinical and histological evidence for ALD has developed[156,157]. Other patient factors that are associated with increased risk of progression to cirrhosis included female sex, tobacco use, binge drinking, obesity and concomitant chronic

viral hepatitis[23,158-162]. Demographic and clinical factors associated with increased mortality in patients with ALD include persistent alcohol use, increasing age, tobacco use, cirrhosis with a higher Child-Pugh score (based on bilirubin, albumin, INR, grade of encephalopathy and ascites), degree of malnutrition, severe deficiency in 25-hydroxyvitamin D, development of cirrhotic complications, and concomitant chronic viral hepatitis infection[163-168].

Overall, patients with alcoholic cirrhosis have a poor five year prognosis. However, alcoholic patients who develop complications from their cirrhosis do significantly worse than those with well compensated alcoholic cirrhosis. In a recent study, patients with well compensated alcoholic cirrhosis had an estimated 5-year mortality rate of approximately 58%. The presence of ascites only increased mortality by 1%, however, patients who developed both ascites and variceal bleeding had a significantly increased 5-year mortality of 80%. Patients who developed hepatic encephalopathy fared the worst with an estimated 5-year mortality of 85%[157]. Patients who develop hepatorenal syndrome (HRS) and who do not receive a liver transplantation, have a dismal prognosis. In a recent study of cirrhotic patients with type 1 HRS, patients with alcoholic cirrhosis had a median survival of only 8 d[169].

The degree of protein-calorie malnutrition (as measured by percent ideal body weight, tricep skin fold thickness, mid-arm muscle circumference, creatinine height index, albumin, transferrin, total lymphocyte count and delayed cutaneous hypersensitivity) in patients with ALD correlates closely with the development of serious complications from liver disease (ascites, encephalopathy, and hepatorenal syndrome), as well as overall mortality[166]. In patients with AH, those with moderate protein malnutrition had a significantly better 6-mo survival rate (75%) than those with severe malnutrition (55%)[166] as well as a significantly better 1-year survival rate (57%) than those with severe malnutrition (24%)[170]. In addition, cirrhotic patients with poor nutrition have a 3-fold greater probability of developing hepatorenal syndrome[171].

There is limited evidence that laboratory studies can have predictive prognostic significance for ALD. However, in a recent study of ALD patients, a decreased α-aminobutyrate/ cystathionine ratio predicted the presence of ALD on liver biopsy and cystathionine levels correlated with the stage of fibrosis in ALD patients[172].

The stage of liver disease is an important prognostic factor for patients with ALD. In a VA study, 281 alcoholic patients were followed prospectively over a 48 mo period and their ALD was staged with liver biopsy. Simple steatosis, an early stage of ALD, was associated with a 30% mortality at 4 years. Alcoholic hepatitis alone, the next stage along the ALD spectrum, carried an estimated 40% 4-year mortality rate. Stable cirrhosis without alcoholic hepatitis carried a 50% mortality rate, and, lastly, the combination of cirrhosis and alcoholic hepatitis was the most deadly and carried the highest mortality rate of approximately 65% at 4 years[173].

Several specific histological findings are important prognostic indices in ALD. For example, the presence of MDB is an important marker of alcoholic related liver injury in alcoholics. MDB have been found in 76% of patients with alcoholic hepatitis and in 95% of patients with concomitant alcoholic cirrhosis[174] and the presence of MDB is associated independently with progression of fibrosis[175]. Pericellular fibrosis, a progression of perivenular fibrosis[176,177], and the presence of ASH on biopsy[178] are also independent predictors of progression to fibrosis and development of cirrhosis in patients with ALD.

In a recent study of Danish men and women with biopsy verified alcoholic steatosis or steatohepatitis, patients with alcoholic fatty liver disease had markedly increased 5 year risk of cirrhosis (6.9%) and mortality (16.7%) compared with a matched reference cohort from the general population (0.3% and 4.3% respectively). In addition, the cirrhosis risk was more than twice as high for patients with steatohepatitis than those with pure steatosis and was higher for women than for men[179]. In another European study of patients with histologically documented ASH, a liver biopsy with the presence of marked intraparenchymal cholestasis was an independent predictor of poor short term outcome in addition to the patient's age and Maddrey's discriminant function score[180].

Several scoring systems have been developed and validated to assess the severity and prognosis of patients with alcoholic hepatitis. Maddrey's discriminant function (MDF), a calculation based on prothrombin time and total bilirubin level (MDF = 4.6 × prothrombin time - control prothrombin time + serum bilirubin), has been used in clinical practice for over three decades to identify patients with severe alcoholic hepatitis who might benefit from corticosteroid therapy[181]. Patients with a MDF score of 32 or greater have been shown to have a high short-term mortality with improved clinical outcomes after receiving corticoidsteroids[182].

The model for end stage liver disease (MELD) score (based on serum bilirubin, creatinine, and INR) was initially developed to predict survival in patients with cirrhosis and was later found to accurately predict short-term survival in patients hospitalized for alcoholic hepatitis with some evidence that it is a better prognostic model for alcoholic hepatitis than the MDF score or Child-Pugh (CP) score and classification[183], which is based on bilirubin, albumin, prothrombin time prolongation, degree of ascites and degree of hepatic encephalopathy. The sensitivity and specificity of the MELD score (12 or greater) for predicting 30-d mortality in ASH has been shown to be 86% and 81% as compared to the MDF score (32 or greater) which has a sensitivity of 86% and specificity of 48%[184]. A higher MELD score cut off value (21 or greater) has been shown to have improved sensitivity (75%) and specificity of (75%) for predicting 90-d mortality in AH[185].

The Glasgow alcoholic hepatitis (GAH) score identifies a subgroup of patients with a MDF score of 32 or greater who will recover without corticosteroid therapy[186].

The GAH is a multivariable model that includes age, serum bilirubin, blood urea nitrogen, prothrombin time, and peripheral white blood cell count. In a study of 225 patients with AH and a MDF score of 32 or higher, patients with a GAH score of 9 or greater who received corticosteroids had improved survival rates when compared with those who did not receive therapy (78% *vs* 52% survival at 28 d; 59% *vs* 38% survival at 84 d)[187]. No survival benefit was observed in patients with a GAH score of 8 or less who received early corticosteroid treatment.

The Lille score evaluates a patient's serum bilirubin response to corticosteroid treatment after 7 d and can aid the clinician in determining whether or not to continue corticosteroid therapy for a full 28 d course. The model includes age, albumin, change in bilirubin over 7 d, prothrombin time and creatinine. A score > 0.45 suggests that a patient is not responding to therapy. Interestingly, the Lille model outperformed CP, MDF, GAH and MELD scores in predicting survival at six months[188].

The age, serum bilirubin, INR and serum creatinine (ABIC) score was developed to stratify patients with AH based on their prognosis. Patients were categorized into low, moderate and high risk groups based on their risk of death at 90 d and one year (25%, 70% and 100% respectively). This model could potentially be used in order to identify patients who may benefit from clinical trials. The ABIC score performed equally well as compared to MDF, MELD and GAH in predicting 90-d survival (AUROC 0.80-0.81) in a confirmatory cohort[189].

A recent study evaluated the utility of CP, MELD, MDF, GAH and AIBC scores in predicting short-term and long term survival in 44 patients with histologic confirmation of AH and found that all scores, with the exception of CP, had similar accuracy in predicting short-term prognosis. All models were poor predictors of survival beyond six months with none of the model's AUROC exceeding 0.74[190]. The only factor that was significantly associated with survival after one year was abstinence from alcohol within 3-6 mo of diagnosis of AH (AUROC of 0.83).

CONCLUSION

ALD is a condition that affects only a small percentage of heavy drinkers. The diagnosis of ALD can be challenging and is based on a combination of clinical and laboratory findings in addition to the essential role of communication with the patient to assess the amount and duration of alcohol intake. Clinical findings may be minimal or absent in early ALD characterized only by hepatic steatosis, whereas in cirrhosis there will be typical signs and symptoms of cirrhosis and portal hypertension. Laboratory studies characteristic of ALD include elevated transaminase levels with AST greater than ALT but also increased MCV, GGT, and IgA to IgG ratio.

In most patients, the diagnosis will be established by thorough history, clinical and laboratory findings. However, in uncertain situations, it can be supported by imaging and liver biopsy results. In most cases, the histological features of ALD can ultimately define the diagnosis according to the typical presence and distribution of hepatic steatosis, inflammation, and Mallory-Denk bodies. Consideration should be given to non-invasive methods, including FibroScan and magnetic resonance elastography, which have the potential to diagnose early ALD but they have not been evaluated yet in this condition.

In addition, clinical and laboratory parameters are important for predicting the prognosis of ALD in more advanced and severe cases and for determining the therapeutic approach. Because of the potential reversible nature of ALD with sobriety, regular screening of the general population and early diagnosis are essential.

Currently, there are no clear, uniform definitions available for ASH and alcoholic hepatitis, particularly in the presence of chronic liver disease or cirrhosis. It is unclear if they represent the same entity or if they are different conditions along the spectrum of ALD. The status of ASH and alcoholic hepatitis in the spectrum of ALD represents a gap in current research and an area of needed further investigation.

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REVIEW

Advances in the management of peritoneal mesothelioma

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Abstract

Malignant peritoneal mesothelioma (PM) is an infrequent disease which has historically been associated with a poor prognosis. Given its long latency period and non-specific symptomatology, a diagnosis of PM can be suggested by occupational exposure history, but ultimately relies heavily on imaging and diagnostic biopsy. Early treatment options including palliative operative debulking, intraperitoneal chemotherapy, and systemic chemotherapy have marginally improved the natural course of the disease with median survival being approximately one year. The advent of cytoreduction (CRS) with hyperthermic intraperitoneal chemotherapy (HIPEC) has dramatically improved survival outcomes with wide median survival estimates between 2.5 to 9 years; these studies however remain largely heterogeneous, with differing study populations, tumor biology, and specific treatment regimens. More recent investigations have explored extent of cytoreduction, repeated operative intervention, and choice of chemotherapy but have been unable to offer definitive conclusions. CRS and HIPEC remain morbid procedures with complication rates ranging between 30% to 46% in larger series. Accordingly, an increasing interest in identifying molecular targets and developing targeted therapies is emerging. Among such novel targets is sphingosine kinase 1 (SphK1) which regulates the production of sphingosine-1-phosphate, a biologically active lipid implicated in various cancers including malignant mesothelioma. The known action of specific SphK inhibitors may warrant further exploration in peritoneal disease.

Key words: Peritoneal mesothelioma; Mesothelioma; Hyperthermic intraperitoneal chemotherapy; Cytoreduction; Sphingosine kinase; Sphingosine-1-phosphate; FTY720

Core tip: Peritoneal mesothelioma (PM) historically has been associated with a very poor prognosis. Cytoreduction with hyperthermic intraperitoneal chemotherapy improved survival outcomes but carries significant morbidity. Increasingly, research has focused on identifying molecular targets and only a handful have been described; even fewer directed therapies have been evaluated. We review the role of sphingosine kinase 1 and sphingosine-1-phosphate (S1P) signaling in PM and discuss the possibility of targeting it with FTY720, a functional antagonist of S1P Receptor 1. Further investigation is warranted in this new avenue of interest.

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INTRODUCTION

Epidemiology of peritoneal mesothelioma

Peritoneal mesothelioma (PM) represents the second most common site of malignant mesothelioma and ac-

counts for 10% to 20% of reported cases[1,2]. Of the 10589 patients with mesothelioma identified in the SEER database between 1973 and 2005, 1112 or 10.5% had disease which was abdominal in origin. Modest differences in incidence rates have been reported among Western countries ranging between 0.5 to 3 cases per million[3]. In the United States, the overall incidence has remained unchanged but age adjusted rates have demonstrated a stepwise increase per decade with 15000 cases projected to occur by 2050[3-5]. Males constitute 56% of cases; compared to those with thoracic disease, patients with PM are more likely to be female[6] and younger[7]. No racial predilection has been recognized[2,4].

Asbestos exposure is the strongest known risk factor for the development of malignant mesothelioma[5]. The association with peritoneal disease, while observed in various epidemiological reports, however, is weaker and not conclusive[3,8]. Factors abating this relationship have included long latency periods of 20 to 50 years from exposure to disease and lack of pathogenesis directly implicating asbestos fibers[8,9]. There is some evidence, however, to suggest that cumulative exposure has been associated with increased prevalence. Berry *et al*[10] studied the exposure of crocidolite among miners over several decades. More than 67% of identified cases of peritoneal mesothelioma occurred with an exposure of greater than 50 fibers per mL years; this was in contrast to only 16% subjects who developed disease with less than 10 fibers per mL years of exposure. Likewise, no cases of PM were observed within 20 years of exposure. Various other environmental factors have been implicated and include thorotrast, erionite - volcanic ash, therapeutic radiation, and chronic peritonitis[3,11]. In the absence of environmental exposures, familial Mediterranean fever, mesothelioma genetic susceptibility syndrome with BRCA germline mutations, and simian vacuolating virus have been postulated to contribute to PM[2,12].

Presentation and diagnostic workup

Symptoms and signs are non-specific with most relating increasing abdominal girth, ascites, or pain[12,13]. Other reported findings have included weight loss, fevers, night sweats, early satiety, anorexia, emesis, constipation, and presence of umbilical hernia; an abdominal mass was appreciated in 10%-30%[13,14]. Computed tomography (CT) imaging is the most common initial imaging modality and can reveal moderate to extensive ascites with peritoneal, visceral, or omental involvement. Yan *et al*[15] reported that CT radiographic findings of large tumor burden with significant bowel distortion or the presence of a small bowel obstruction to be predictive of incomplete resectability. MRI imaging may more accurately quantify the extent of disease, however, its routine use is not supported yet[16]. The role of PET scan is not well defined and may be have some limited use in the detection of recurrent disease[17]. Biopsy is required to establish a diagnosis and can be performed radiographically or surgically. Paracentesis with fluid cytology has a variable sensitivity of 32% to 76% with the major limitation being difficulty in distinguishing benign from malignant lesions[2,18]. Various serum tumor markers have also been explored. CA-125 and CA15.3 were noted to be elevated at baseline in 53.3% and 48.5% of patients; their role, however, may be more important in monitoring disease recurrence or progression than at initial diagnosis[19,20].

TREATMENT

Outcomes of conventional treatment

Traditional treatments for peritoneal mesothelioma have historically yielded modest survival ranging between 6 and 16 mo with median survival being approximately one year[21,22]. Operative therapies have largely centered around palliative cytoreduction. Rogoff *et al*[23] reported one of the earliest series with 6 of 12 patients undergoing debulking with a median survival being no greater than 13 mo for the entire cohort. More recent series have reported similar median survival periods of one year[13,24,25]; compared with operative biopsy alone, however, debulking offered a modest survival improvement of 7 mo[24]. The use of conventional systemic chemotherapy likewise has not greatly impacted the natural course of PM with response rates between 11% and 28%[26-28]. More recent novel cytotoxic agents like premetrexed have improved response rates to as high as 37% with median survival ranging between 7.6 and 12.1 mo with dual agent regimens; Simon *et al*[29] reported an improved survival period of 26.8 mo with gemcitabine combination therapy in 20 patients but regimen was accompanied with a 60% incidence of grade 3 or 4 neutropenia[27-29]. Monotherapy with intraperitoneal chemoinfusion has likewise not offered any significant benefit with reported outcomes of 9 to 12 mo[30,31].

Cytoreduction with hyperthermic intraperitoneal chemotherapy

Cytoreduction (CRS) with hyperthermic intraperitoneal chemotherapy (HIPEC) has been more widely adopted over the last 15 years and has been campaigned as the standard of care for patients with operable disease[1]. Ma *et al*[32] was the first to report its technical efficacy in ten patients in 1997; he reported no treatment related mortality and symptomatic ascites palliation in 7 patients. Two years later, Park *et al*[33] reported on 18 patients undergoing CRS + HIPEC with a two year survival rates of 80% and progression free survival of 26 mo. In studies reporting at least 2 year outcome data, median survival has varied greatly ranging between 29.5 and 100 mo and five year overall survival between 30%-90% when reported (Table 1). The wide range of these findings reflects the heavily heterogeneous nature of differing study populations, eras of treatment, institutional follow-up protocols, chemotherapy regimens, operative techniques, and tumor biology. The most numerous reports have been coordinated or originated mainly from two principal centers: the National Cancer Institute of Milan (Milan, Italy) and

the Washington Cancer Center (Washington, DC, United States). Larger series, however, have relied on appropriate collaborative approaches[20,34-45]. Yan *et al*[42] published the largest longitudinal series with 405 patients in a multi-institutional review between 1989 and 2009. Here, 92% of patients received HIPEC most commonly with cisplatin and doxorubicin and an additional 23% subsequently received early post-operative chemotherapy between postoperative day 1 and 5, most commonly with paclitaxel. Overall median survival was 53 mo and five year survival was 47%. Such multi-institutional registries have gone onto be utilized in assessing large-population based prognostic factors[43,46,47].

Reports describing long term survival at 10 years are slowly emerging. Two studies listed survival estimates as secondary outcomes and ranged between 26% and 44.6%[35,47]. Baratti *et al*[34] reported on 10 year prognosis in 108 patients undergoing complete cytoreduction and HIPEC at two centers. Chemotherapy regimens included cisplatin and doxorubicin in the vast majority of patients and cisplatin or mytomycin-C in six. At five and ten years, overall estimates were 52.4% and 44.6%, respectively, and median survival was 63.2 mo.

The most optimistic, large series survival estimates were reported by Feldman *et al*[48] in 49 patients. More than half of patients had previously undergone debulking surgery, and thereafter, all subjects underwent HIPEC with cisplatin. Thirty five patients or 72% additionally received a single postoperative intraperitoneal (IP) dose of fluorouracil and paclitaxel. Median overall survival was 92 mo and 1, 3, and 5 year survival was 86%, 59%, and 59%, respectively. Worse survival was associated with deep tumor invasion, age greater than 60 years, absence of prior debulking, and residual disease greater than 1 cm. Of note, 36% of patients had lower grade histology which impacted survival on univariate analyses. Baratti *et al*[36], likewise, reported an exceptional 5 year survival estimate of 90% with median survival not being reached; however, this study sought to describe borderline malignant subtypes of PM in 12 patients.

Chemotherapy agents used for HIPEC

No randomized clinical trials exist that assess which HIPEC chemotherapy regimen is superior. The majority of retrospective reports have described cisplatin mono- or dual therapy (Table 1). Blackham *et al*[49] investigated 19 patients receiving monoagent mitomycin and 15 receiving cisplatin in a retrospective review; he found patients administered cisplatin were more likely to be alive at 1, 2, and 3 years with a nearly 30 mo median survival advantage. Similarly, Alexander *et al*[47] identified the use of cisplatin over mitomycin-C to be associated with favorable survival; this however was noted only in optimally cytoreduced patients. The choice of agents seems largely driven by an institution's experience more so than empiric evidence.

The role of peri-operative systemic chemotherapy in addition to HIPEC with CRS was examined retrospectively by Deraco *et al*[50] in 90 patients. Sixty of these patients received preoperative chemotherapy most commonly with a platinum-based agent and premetrexed or gemcitabine; 12 patients underwent triple agent therapy and two received more than three drugs. An additional 30 patients naïve to systemic therapy received post-operative treatment with platinum with premetrexed or gemcitabine. These cohorts were compared to 26 patients who underwent a logoregional approach only. No significant difference was observed in overall survival among groups with the survival estimate being 49% at 5 years for the entire series. A trend towards improved progression free survival, however, was observed in those receiving preoperative treatment, and overall 3 year survival favored those treated with preoperative pemetrexed and platinum chemotherapy (63% *vs* 42%-48%, non-significant). No differences in prognostic factors were identified among groups and the epithelioid histological was most common subtype. Yan *et al*[42] similarly reported on 22 patients receiving pemetrexed dual agent therapy after cytoreductive surgery and demonstrated no significant influence on survival. To date, combination regional and systemic therapies for PM remain largely unexplored.

Role of aggressive cytoreduction regimens

The extent of cytoreduction has repeatedly shown to impact survival[40,51,52]; a handful of studies have gone onto better define the role of aggressive cytoreduction. The underlying principle of cytoreduction is to remove all the macroscopic disease and use HIPEC to address any remaining microscopic disease[1]. Baratti *et al*[53] attempted to address the benefit of patients undergoing resection of peritoneum free of gross disease in addition to macroscopic disease. In a case-control study, 30 patients undergoing selective resection of macroscopic disease were compared to a cohort of 30 individuals undergoing "complete" parietal peritonectomy, which included abdominal regions uninvolved by disease. The five year overall survival was significantly greater at 63.9% *vs* 40% in the "complete" resection group. The median overall survival was not reached in the "complete" group despite a follow-up of 50.3 mo and was 29.6 mo in the selective resection group. Progression free survival was likewise significant being 54.3% *vs* 24.9% in favor of more aggressive peritonectomy. Interestingly, "complete" resection carried no significant increase in operative risk and was associated with a shorter length of stay by 8 d. A subsequent pathologic review revealed peritoneal disease involvement in 54% of samples deemed grossly negative at exploration which may warrant more aggressive cytoreduction approach.

More recently, previously abandoned and multi-stage modalities have been re-explored with the use of CRS and HIPEC. Wong *et al*[52] addressed the outcomes of repeated CRS with HIPEC. Twenty six of 29 patients underwent debulking with cisplatin-based HIPEC. Eight or 31% then went on to have one or more repeated HIPEC procedures. The median overall survival for the re-opera-

Table 1 Selected studies examining overall survival with cytoreduction and hyperthermic intraperitoneal chemotherapy

Ref.	Year	Era	Sample No.	Age	Gender (female)	HIPEC Agents	Histological Subtype or Grade	Follow-up	Median survival	1 yr	2 yr	3 yr	5 yr	7 yr	10 yr
Park *et al*[33]	1999	1993-1998	18	47	28%	CDDP	9 E, 3 mixed 1 MMF 1 cystic 4 Unk	19	NR		80				
Loggie *et al*[112]	2001		12	51[1]	8%	MMC	Not stated	45.2	34.2	60	60	50	33	33	
Feldman *et al*[48]	2003	1993-2002	49	47	43%	CDDP	26 E, 4 S 16 TP 1 adenomatoid	28.3	92	86	77	59	59		
Costamagna *et al*[61]	2003	1995-2003	24			CDDP + DOXO, single agent MMC or DOXO			40	78	70	70	52		
Brigand *et al*[6]	2006	1989-2004	15	53.6	29%	CDDP + MMC	12 E, 2 B 1 MC	46.7	35.6	69.3	57.7	43.3	28.9		
Elias *et al*[60]	2007	1996-2005	26	46[1]	46%	oxaliplatin + irinotecan, single agent oxaliplatin or DOXO	13 E, 1 B 11 TP, 1 MC	55	100	88	83	68	63		
Gómez Portilla *et al*[113]	2007	1998-2005	7	50[1]	43%	CDDP + DOXO, single agent MMC or oxaliplatin	5 E, 2 B	11	NR	43	43	43	43		
Hesdorffer *et al*[55]	2008	1997-2000	27	53[1]	26%	CDDP + MMC	23 E, 4 S		70			67%			
Passot *et al*[114]	2008	1989-2006	22			CDDP + MMC	16 E, 3 B 3 MC	47	36.9	69	62	52	31		
Chua *et al*[115]	2009	1997-2008	20	55.7[1]	30%	CDDP + DOXO	16 E, 1 B, 2 S 1 MC	18.1	29.5	78.2		46.3			
Blackham *et al*[49]	2010	1993-2008	34	54.9[1]	32%	CDDP or MMC	29 E, 4 B 1 Unk	72	40.8	61		56	17		
Kluger *et al*[54]	2010	1997-2004	47	49[1]	36%	CDDP + MMC	43 E, 4 B	54	54.9	80.9		61.7	48.9		
Cao *et al*[46]	2012	1989-2009	294	50	46%	CDDP + DOXO[2]	259 E, 27 B/S 8 Unk	25	67	83	62	52			
Alexander *et al*[47]	2013	1992-2010	211	52	60%	CDDP or MMC	113 High, 54 Low 44 Unk		38.4				41		26
Schaub *et al*[59]	2013	1994-2010	104	50.9	39%	CDDP	90 E, 14B/S	49.4	52			58	46		
Wong *et al*[52]	2013	2004-2012	26	64	38%	CDDP	15 E, 3 B 1 MMF 3 WD, 1 cystic 3 Unk		41.2						
[NM]Deraco *et al*[37]	2003	1995-2002	19	49[1]	53%	CDDP + MMC or DOXO	13 E, 1 S, 1 mixed 2WD, 2MC	27	NR			69			
[NM]Deraco *et al*[39]	2003		28		61%								70		
[NM]Deraco *et al*[38]	2003		61	51[1]	49%	CDDP + MMC or DOXO		20					54		
[NM]Deraco *et al*[40]	2006	1995-2005	49	52[1]		CDDP + DOXO or MMC	43 E, 6 B	20.3[1]					57		
[NM]Deraco *et al*[50]	2013	1995-2011	116	54.4[1]	48%	CDDP + DOXO or MMC	105 E, 11 B/S	32.9[1]					49		
[NM]Baratti *et al*[20]	2007	1997-2005	60	53.5	60%		43 E, 6 B 5 MC, 6 P	23					53.7		
[NM]Baratti *et al*[36]	2007	1995-2006	12	38	100%	CDDP + DOXO	4 MC, 8 WD	27	NR				90		

[NM]Baratti *et al*[35]	2010	1996-2008	83	54	55%	CDDP + MMC or DOXO	72 E, 10 B, 1 S	52	44				49.5		45.5
[NM]Baratti *et al*[34]	2013	1996-2012	108	56.5	46%	CDDP + DOXO or MMC	93 E, 14 B, 1 S	48.8	63.2				52.4		44.6
[WC]Yan *et al*[45]	2006		100	50[1]	40%	CDDP + DOXO	86 E, 7 B/S 7 Unk	48	52	78		55	46	39	
[WC]Yan *et al*[44]	2007	1989-2005	70	47[1]	43%	CDDP + DOXO	65 E, 5 B	35	59	82	67	57	49		
[WC]Yan *et al*[41]	2007	1989-2005	62	47[1]	45%	CDDP + DOXO	57 E, 5 B	37	79	84		58	50		
[WC]Yan *et al*[42]	2009	1989-2009	401	50[1]	44%	CDDP + DOXO[2] or MMC, single agent CDDP or MMC	318 E, 48 B/S 35 Unk	33	53	81		60	47		
[WC]Yan *et al*[43]	2011	1989-2009	294	50[1]	46%	CDDP + DOXO[2]	259 E, 27 B/S 8 Unk	24	67	83	62	52			

[1]Mean values are indicated with an asterisk. Follow-up and survival are reported in months; [2]Most common chemotherapy agent reported. The principal centers including the National Cancer Institute of Milan and the Washington Cancer Center are noted with superscripts NM and WC, respectively. NR: Not reached; CDDP: Cisplatin; MMC: Mitomycin or Mitomycin C; DOXO: Doxorubicin; E: Epithelial or epithelioid subtype; B: Biphasic; S: Sarcomatoid or sarcomatous; B/S: Biphasic or sarcomatoid; MMF: Malignant or epithelial with mucinous features; MC: Benign multicystic; TP: Tubillopapillary; P: Papillary or mixed papillary; WD: Well-differentiated; Unk: Unknown or not specified.

tion group was far superior at 80 mo compared to 27.2 mo in the single treatment cohort. The median time to the second operation was 15.6 mo and most (77%) received early postoperative chemotherapy with Taxol and 5-fluorouracil. Both groups otherwise had similar completeness of cytoreduction scores, demographics, and similar overall number of complications. Kluger *et al*[54] reported on two-stage operative cytoreduction with intraperitoneal chemotherapy in 47 patients. Subjects initially underwent partial cytoreduction with peri-operative intraperitoneal therapy with single or dual regimens of cisplatin, gemcitabine, doxorubicin, or gamma interferon. A second laparotomy with CRS and HIPEC was performed in 35 using cisplatin and mitomycin C; median survival was 54.9 mo with 1, 3, and 5 year overall survival being 81%, 62% and 49%, respectively. Hesdorffer *et al*[55] reported on multi-modality treatment in 27 patients who underwent operative debulking with post-operative IP therapy followed by HIPEC with mitomycin and cisplatin and then followed by whole abdominal radiation between 3000 and 3080 cGy. Overall median survival was 70 mo and three year survival was 67%. The retrospective nature of these reviews limits drawing any firm conclusions, but a multi-modality approach may offer the most aggressive treatment for patients with PM.

Role of laparoscopy

Diagnostic laparoscopy with biopsy has been previously described as a safe alternative in obtaining a histological diagnosis[13,56]. Its role in assessing resectability before CRS with HIPEC in PM was explored in 33 patients. Patients with potentially resectable disease on pre-operative imaging underwent exploration. Ninety one percent of patients were deemed likely to obtain complete cytoreduction; of these, only one patient was not on subsequent laparotomy, yielding an overall specificity of 75% and accuracy of 97%[57].

Prognostic factors in CRS with HIPEC

More than half of the studies reporting on prognostic factors have reported completeness of cytoreduction to be associated with improved survival on multivariate analyses[35,38,40,42,43,45,50-53]. Nodal status, histological subtype, nuclear grade, and mitotic count have also been cited[34,35,40,42,43,45,47,51-54] (Table 2). Concordant findings were reported in the large multi-institutional series by Yan *et al*[42]. Interestingly, 29 patients did not receive HIPEC; a subsequent multivariate sub-analysis demonstrated that HIPEC correlated with improved survival. Baratti *et al*[53] similarly identified another surgical factor, "complete" peritonectomy, as positive influencing survival.

Female gender was shown by Cao *et al*[46] to be among patient factors to positively influence survival; the female cohort accounted for 46% in the study population and was more likely to have lower peritoneal cancer indices and earlier stage compared to males[41,46]. Interestingly, the presence of the nuclear estrogen receptor beta was shown to be an independent predictor of survival in peritoneal disease[58]. Schaub *et al*[59] found pre-operative CA-125 to influence survival; a prognostic nomogram was proposed by incorporating this marker along with peritoneal cancer index and histological subtype as clinical assessment tool for 3 and 5 year survival; positive predictive values have been reported as 73.1% and 73.9% respectively.

Table 2 Prognostic factors in cytoreduction and hyperthermic intraperitoneal chemotherapy procedures

Ref.	Year	Sample No.	Prognostic factors overall survival (multivariate only)
Deraco *et al*[38]	2003	61	Completeness of cytoreduction
Feldman *et al*[48]	2003	49	[1]No prior debulking, deep invasion, age > 60, residual disease > 1 cm
Nonaka *et al*[51]	2005	35	Completeness of cytoreduction, low mitotic count, lower nuclear grade
Deraco *et al*[40]	2006	49	Completeness of cytoreduction, low mitotic count/50 HPF
Yan *et al*[45]	2006	100	No lymph node metastasis, female gender, epithelial type, adequate cytoreduction
Baratti *et al*[20]	2007	60	[1]High-grade histology, WHO performance status > 0, Inadequate cytoreduction
Yan *et al*[41]	2007	62	Mesothelioma nuclear size
Yan *et al*[42]	2009	401	Epithelial subtype, absence of lymph node metastasis, completeness of cytoreduction 0/1, HIPEC
Baratti *et al*[35]	2010	83	Pathologically negative lymph nodes, epithelial subtype, mitotic count ≤ 5/50 HPF, Completeness of cytoreduction
Kluger *et al*[54]	2010	47	[1]Biphasic histological subtype
Yan *et al*[43]	2011	294	[1]Biphasic/sarcomatoid subtype, completeness of cytoreduction score of 2/3, proposed TNM Stage Ⅱ or Ⅲ
Cao *et al*[46]	2012	294	Female gender, TNM staging
Baratti *et al*[53]	2012	60	Complete parietal peritonectomy, complete cytoreduction, negative lymph nodes, Epithelial histology, low MIB-1 index
Alexander *et al*[47]	2013	211	Age < 60 yr, R0-1 *vs* R2-3, low histologic grade, use of cisplatin *vs* mitomycin-C
Baratti *et al*[34]	2013	108	Epithelial histology, histologically negative lymph nodes, Ki-67 < 10%
Deraco *et al*[50]	2013	116	Histological subtype, completeness of cytoreduction, absence of morbidity 3-5 grade
Schaub *et al*[59]	2013	104	Histological subtype, pre-CRS PCI, preoperative serum CA-125
Wong *et al*[52]	2013	29	Lower peritoneal carcinoma index, completeness of cytoreduction
Pillai *et al*[58]	2013	33	Presence of nuclear estrogen receptor beta

[1]Variables that have negatively impacted overall survival.

Morbidity and peri-operative mortality

Overall morbidity rates have varied widely between 14% and 71% (Table 3). Larger series with at least 50 subjects have reported 28%-41% incidence of grade 3 or greater complications[35,42,44,45,50,53]. When reported, peri-operative mortality has ranged between 1% and 11% and re-operation rates up to 20% with the most common indication being hemorrhage[6,34,37,42,44,46,47,52,60-62]. Complications related to fistula formation, perforation, dehiscence, abscess formation are significant and in large series have been reported in up to 18% of cases[42]. Cardiopulmonary complications are the second most frequently encountered followed by those related to infection. It remains difficult to distinguish those complications stemming from operative intervention and those related to chemotherapy regimens. In terms of long-term survival, Deraco demonstrated worse survival with patients with grade 3 to 5 complications[50]. Length of stay has been investigated in a handful of reports and has ranged from 9 to 41.5 d; Wong *et al*[52] reported on a median stay of only 8 d for patients undergoing repeat HIPEC. Surprisingly, morbidity rates only have a weak association with the duration of inpatient admission; other factors such as administration of early post-operative chemotherapy may be involved but are not reported.

FUTURE DIRECTIONS; MOLECULAR TARGETED THERAPY

Investigated molecular targets and therapeutic agents

A variety of molecular targets have been identified in PM and of these, a handful of respective therapeutic agents have been investigated. Foster *et al*[63] discovered mutations in the epidermal growth factor receptor (EGFR) in a subset of 29 patients, which were associated with a higher rate of optimal cytoreduction and a trend towards improved 3 year overall and progression free survival. Erlotinib, an EGFR inhibitor, was then investigated using COS-7 cell lines transfected with mutant EGFR at different drug concentrations along with EGF; based on subsequently decreased EGFR phosphorylation, it was postulated that erlotinib may warrant further exploration. Kalra *et al*[64], however, questioned any wide spread role of EGFR targeted therapies because none of 33 peritoneal mesothelioma tumors he interrogated expressed EGFR sensitizing mutations.

Varghese *et al*[65] identified up-regulation in genes related to the phosphatidylinositol-3 kinase (PI3K) and the mammalian target of rapamycin (mTOR) signaling pathways to be associated with poorer survival among 41 patients undergoing CRS and HIPEC. Under-expression was associated with an 80% 3 year survival with a median period of 69.5 mo compared with 47.4 mo for the entire cohort and 24 mo for over-expressers. Using an *in vitro* model, cells were treated with a dual PI3K and mTOR inhibitor, NVP-BEZ235, demonstrated significant suppression of cell proliferation[65].

Mesothelin, glycosylphosphatidylinositol-anchored glycoprotein, has also been recognized to be highly expressed in malignant mesothelioma along with pancreatic, ovarian, and some lung cancers. Three agents targeting mesothelin have been tested to date; SS1P, a recombinant immunotoxin targeting mesothelin; MORAb-009, a chimeric anti-mesothelin monoclonal antibody; and CRS-207, a live-attenuated Listeria monocytogenes vector encoding human mesothelin[66]. Of these, SS1P has undergone phase I testing in 24 patients including five with peritoneal mesothelioma and has shown short term resolution of ascites in one patient[67,68]. The second trial

Table 3 Morbidity, peri-operative mortality, and length of stay associated cytoreduction with hyperthermic intraperitoneal chemotherapy

Ref.	Year	Sample No.	Complication rate	Peri-operative mortality	Re-operation	Abdominal	Cardiac/ pulmonary	Sepsis	Wound Infection	Other infection	Renal	Vascular	Hematologic	Other	LOS
Park *et al*[33]	1999	18	24%		1	1	1	1	2	1				1	
Feldman *et al*[48]	2003	49	25%	0	2	4	5	2	3	1	1			2	
Costamagna *et al*[61]	2003	24	26%	11											
Brigand *et al*[6]	2006	15	40%	0	0					0	0			0	16.3
Elias *et al*[60]	2007	26	54%	4	4	1	1			4	1	3	2	2	28[1]
Gomez *et al*[113]	2007	7	71%												41.5[1]
Hesdorffer *et al*[55]	2008	27	30%	0		2	1			1		1		3	
Passot *et al*[114]	2008	22	47%	0											
Chua *et al*[115]	2009	20	65%	1		2	5	2			1		1	3	16.5[1]
Yano *et al*[62]	2009	17	41%	5.8			2			2		1		3	
Kluger *et al*[54]	2010	47	34%	2	1	3	9	2	2	9	2	1		1	16[1]
Cao *et al*[46]	2012	294	33%	2		50	38				26		15		23[1]
Alexander *et al*[47]	2013	211	30%	2.3	20	20	25		9			6	8	11	11
Wong *et al*[52]	2013	29	65%	4	1	4	1		6		2		10	4	9
Deraco *et al*[37]	2003	19	25%	0		3				1	1			3	32[1]
Deraco *et al*[39]	2003	28	14%	0											
Deraco *et al*[38]	2003	61	23%	0		7									
Deraco *et al*[40]	2006	49	15%[G3]			9	2	4		5	4		3	5	24[1]
Deraco *et al*[50]	2013	116	41%[G3]	2.6											25
Baratti *et al*[36]	2007	12	8%[G3]	0	1	1									
Baratti *et al*[116]	2008	5	20%[G3]	0	1	1									
Baratti *et al*[35]	2010	83	28%[G3]	2.4											
Baratti *et al*[117]	2010	12	8%[G3]	0											18
Baratti *et al*[53]	2012	60	28%[G3]	0	7	8	4	3	1		7	2	5	1	
Baratti *et al*[34]	2013	108	39%	1.9		14	10	6			10		7		
Yan *et al*[45]	2006	100	36%[G3]	5		6	8	7		11		5	8	10	22[1]
Yan *et al*[44]	2007	70	41%[G3]	3	4	3	4	3		2		4	5	3	23[1]
Yan *et al*[42]	2009	401	46%[G3]	2		74	57				39		25		22[1]

[1]Mean values. Cells with the superscript G3 represent grade 3 or above complications. Abdominal complications include perforation, abscess formation, dehiscence, and fistula. "Other" complications include neurological and musculoskeletal complaints, emesis, and dehydration. Numerical values represent cases or patient number unless otherwise stated.

demonstrated a partial response with SS1P in two of 12 patients with PM[67,68]. Hassan *et al*[69] reported on a regimen of SS1P, pentostatin, and cyclophosphamide in eleven patients with mesothelioma; two patients with peritoneal disease had a significant tumor reduction up to 8 and 14 mo, respectively. More recent studies have reported on newer mesothelin-targeted agents including the immunocytokine IL12, which has shown comparable anti-tumor activity to SS1P in a murine model of PM[70].

Molecular targets on the horizon include MUC1, a glycoprotein associated with various cancers including breast, colon, and pancreatic adenocarcinoma; recently, Pillai demonstrated that MUC1 was expressed in 90% of patients with PM and may have some prognostic value in predicting poorer survival[71]. Bromelain, a complex of proteolytic enzymes, has been postulated to target glycoproteins including MUC1, and initial experiments have demonstrated that chemo-resistant peritoneal mesothelioma cells lines have increased chemotherapy sensitivity with bromelain combination therapy[72]. Some skepticism exists as no studies have directly examined the effect of this agent on MUC1 in peritoneal disease.

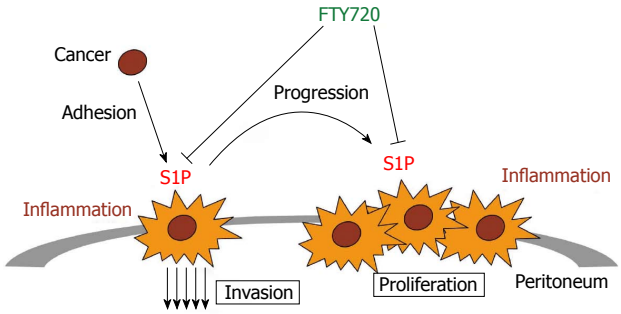

Figure 1 Role of sphingosine 1-phosphate in peritoneal mesothelioma. Inflammation is critical to the development of peritoneal and sphingosine 1-phosphate (S1P) plays an essential role linking it to cancer. Targeting S1P, therefore, with agents like the pro-drug FTY-720 may suppress this process.

Sphingosine kinase 1 as a novel target for mesothelioma

Sphingosine kinase 1 (SphK1) is the lipid kinases that phosphorylate sphingosine to generate sphingosine-1-phosphate (S1P), a lipid mediator. S1P is an important bioactive lipid that has been implicated in multiple physiologic and pathologic processes such as, inflammation, atherosclerosis, asthma, osteoporosis, diabetes, obesity, and particularly cancer, due to its role in cell survival, proliferation, migration, angiogenesis, and lymphocyte trafficking[73,74].

The molecular functions of S1P can be divided into its intracellular action and extracellular signaling, which is coined "inside-out" signaling[73,74]. Intracellular S1P can directly regulate its target proteins, which are histone deacetylases (HDACs) and the E3 ubiquitin ligase tumor-necrosis factor (TNF) receptor-associated factor 2 (TRAF2)[75,76]. Through the regulation on these proteins, S1P involves epigenetic regulation of gene expression of NF-κB signaling, which play key roles in cancer biology. As for the inside-out signaling of S1P, transporters such as ABC transporters and Spns2 have been identified[77,78]. These transporters allow S1P to be exported outside the cell and act as a ligand on membranous five S1P specific G-protein coupled receptors (S1PR1-5), which activate multiple downstream signaling pathways regulating cell differentiation, migration, and survival in an autocrine, paracrine, and/or endocrine manner[79].

Owing to the role of S1P in cancer cells, studies investigating SphK1 as an oncogene have steadily increased[80]. It has been known that S1P possesses a strong angiogenic property[81]. Considering a critical role of lymphangiogenesis in cancer progression[82], our group reported on a new aspect of the SphK1/S1P axis by its involvement in breast cancer-induced lymphangiogenesis which precedes breast cancer metastasis[83,84]. Recently, we have also further demonstrated the indispensable role of the SphK1/S1P axis in colitis and colitis-associated cancer[85-87].

The role of the SphK1/S1P axis in mesothelioma has not been reported only until recently. Kalari *et al*[88] demonstrated elevations in SphK1 expression in both the epithelial and sarcomatoid subtypes of human pleural mesothelioma compared with non-tumor specimens. They further delineated the function of SphK1 *in vitro* and *in vivo*. Examining mesothelioma cell lines, SphK1 mRNA and protein expression were higher in malignant cells, and this over-expression correlated with cellular proliferation. The possible mechanism was S1P regulation on expression of cell cycle-related genes *via* histone acetylation. The SphK2 isotype was not implicated in tumorigenesis.

Using the SphK1 inhibitor, SphK-I$_2$, or gene silencing, S1P production and cell proliferation were likewise reduced. The authors additionally conducted an alternative *in vivo* model in which they exposed the peritoneal lining of mice to mesothelioma inducing agents, specifically long multiwalled carbon nanotubes (MWNT). Exposure to MWNTs have been reported to cause development of granulomas in p53-knockout mice[89]. Compared to wild type mice, the Sphk1 knockout mice demonstrated significantly less MWNT-induced granulomatous inflammation. This result suggested the *in vivo* role of SphK1 as promoting mesothelioma development.

Recently, studies investigating agents targeting S1P signaling have been tested in various settings[90]. Among these, FTY720 has shown some promise; FTY720 (Fingolimod; trade name Gilenya, Novartis) is a FDA-approved drug for treating relapsing forms of multiple sclerosis[91]. It has been shown FTY720 acts as a pro-drug which is mainly phosphorylated *in vivo* by SphK2[92-94]. The phospho-FTY720 mimics S1P action by binding to S1PR1 which is then internalized and degraded[95,96]. S1PR1 signaling, itself, is important for lymphocyte egress from thymus and secondary lymphoid organs to the periphery[97,98]. The down-regulation of S1PR1, therefore, through the known action of FTY720, is considered as immunomodulatory by inducing lymphopenia without generalized immunosuppression[96,99]. In addition to the immunosuppressant property, several reports about FTY720 as an anti-cancer drug in various malignancies have rapidly accumulated[100,101]. We recently reported, in a murine colitis-associated colon cancer model, the administration of FTY720 dramatically reduced tumor size, multiplicity, and tumor load *via* the reduction of SphK1 and S1PR1 expression[85,86]. Others have gone onto also characterize FTY720 as a SphK1 inhibitor in multiple cancer cell lines[102-104]. In hematopoietic malignancies or lung cancer, FTY720 acts as an activator of tumor suppressor protein phosphatase 2A (PP2A) and shows promising preclinical activity[105-108]. In hepatocellular carcinoma, FTY720 was found to decrease recurrence after liver transplantation *via* down-regulation of S1PR1[109]. FTY720 was additionally suggested in combinational therapy with sunitinib for breast cancer, with milatuzumab for lymphoma, and radiotherapy for prostate cancer[103,110,111]. Taken together, targeting S1P signaling by FTY720 might be a potential strategy for pharmocotherapeutics for peritoneal mesothelioma (Figure 1).

CONCLUSION

Peritoneal mesothelioma remains a rare, infrequent disease which historically has been associated with a poor

prognosis. Demonstrable improvements in survival have been made with the wider employment of cytoreduction and HIPEC and a generally more aggressively-focused treatment regimen. Yet despite these advances, significant morbidity still persists and a few options exist for those not amenable to operative intervention. Novel molecular targets such as SphK1 have only recently been associated with PM and represent a potentially promising venue for drug therapy in the future.

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REVIEW

Bovine immunoglobulin protein isolates for the nutritional management of enteropathy

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Abstract

The gastrointestinal tract is responsible for a multitude of digestive and immune functions which depend upon the balanced interaction of the intestinal microbiota, diet, gut barrier function, and mucosal immune response. Disruptions in one or more of these factors can lead to intestinal disorders or enteropathies which are characterized by intestinal inflammation, increased gut permeability, and reduced capacity to absorb nutrients. Enteropathy is frequently associated with human immunodeficiency virus (HIV) infection, inflammatory bowel disease, autoimmune enteropathy, radiation enteritis, and irritable bowel syndrome (IBS), where pathologic changes in the intestinal tract lead to abdominal discomfort, bloating, abnormal bowel function (*e.g.*, diarrhea, urgency, constipation and malabsorption). Unfortunately, effective therapies for the management of enteropathy and restoring intestinal health are still not available. An accumulating body of preclinical studies has demonstrated that oral administration of plasma- or serum-derived protein concentrates containing high levels of immunoglobulins can improve weight, normalize gut barrier function, and reduce the severity of enteropathy in animal models. Recent studies in humans, using serum-derived bovine immunoglobulin/protein isolate, demonstrate that such protein preparations are safe and improve symptoms, nutritional status, and various biomarkers associated with enteropathy. Benefits have been shown in patients with HIV infection or diarrhea-predominant IBS. This review summarizes preclinical and clinical studies with plasma/serum protein concentrates and describes the effects on host nutrition, intestinal function, and markers of intestinal inflammation. It supports the concept that immunoglobulin-containing protein preparations may offer a new strategy for restoring functional homeostasis in the intestinal tract of patients with enteropathy.

Key words: Immunoglobulins; Plasma proteins; Inflammation; Gut barrier; Diarrhea; Malabsorption; Treatment; Nutrition

Core tip: This review article summarizes previous preclinical and clinical studies with serum- or plasma-derived protein preparations with an emphasis on potential benefits for intestinal health and recovery from intestinal disorders. Specifically, how serum-derived bovine immunoglobulin/protein preparations may be useful in restoring intestinal homeostasis (*e.g.*, gut barrier function, immune regulation) following episodes of enteropathy associated with various human disease conditions, such as human immunodeficiency virus infection, inflammatory bowel disease, or irritable bowel syndrome.

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INTRODUCTION

The intestinal epithelium is contiguous with the external environment and adaptively organized to both amplify surface area for nutrient absorption and provide a barrier against harmful microorganisms and toxins[1]. The lumen of the gastrointestinal (GI) tract is occupied by a complex assortment of microbial species, the gut microbiota, which changes in response to diet, age, disease, and medical or pharmaceutical intervention[2,3]. This complex array of bacteria and other microbes exist in symbiosis with the intestinal mucosa and play an important role in nutrient absorption, immune regulation, and gut barrier function. At the same time, a variety of immune and physiological adaptations exist within the GI tract to maintain constant vigilance against potentially harmful pathogens and luminal antigens, while preventing the development of uncontrolled inflammation[4].

A variety of factors, both host-related and environmental, can disrupt intestinal homeostasis and lead to development of intestinal disorders or enteropathies. Such enteropathies are characterized by inflammation in the epithelium and lamina propria of the intestine and occur in association with a variety of human conditions or disease states, including: gluten-sensitivity[5], protein-losing enteropathy[6], environmental enteropathy[7,8], radiation-induced enteropathy[9], drug-associated enteropathy[10], irritable bowel syndrome (IBS)[11,12], inflammatory bowel disease (IBD)[13], and human immunodeficiency virus (HIV) infection[14]. In the small intestine, pathologic mucosal changes include blunting of villi and deepening of the crypts, both associated with inflammation that reduces absorptive capacity and tends to increase gut permeability. In the colon, inflammatory changes may be associated with overt epithelial damage or loss. Clinical signs associated with inflammatory mucosal changes may include abdominal pain or discomfort, nausea, bloating, and abnormal bowel function (*e.g.*, urgency, diarrhea, constipation). The pathophysiologic mechanisms leading to enteropathy are not well understood but may involve the effects of exposure to luminal antigens, toxins, or alterations in intestinal microbiota, as well as host diet, genetics, or dysregulated immune responses.

Certain genetic mutations may also predispose to conditions that are associated with enteropathy. For example, more than 200 polymorphisms have been linked to Crohn's disease. These polymorphisms may predict disease manifestations (stenosis, fistulization, or inflammation), location, and need for surgery[15]. The mutations fall broadly into categories reflecting toll-like receptor-mediated bacterial recognition, autophagy, organic cation transport, lymphocyte differentiation, and barrier function[16,17]. There is some evidence that genetic polymorphisms also play a role in ulcerative colitis[18,19] and IBS[20]. Metabolomic studies have found that malabsorption leads to depleted levels of alanine, glutamine, glutamic acid, isoleucine, leucine, valine, choline and select dietary organic acids, including formate, lactate and succinate, in patients with ulcerative colitis[19,21]. Interestingly, altered expression of genes involved in the production of metabolites from tryptophan through the kynurenine pathway has also been associated with IBS. Clarke *et al*[22] found significantly higher kynurenine: tryptophan ratios in IBS subjects compared to controls, and acute depletion of tryptophan has been associated with higher levels of abdominal pain and lower levels of serotonin production in IBS[23,24]. Altered tryptophan metabolism through the kynurenine pathway has also been implicated in the events leading to intestinal inflammation in HIV-infected individuals[25] and in IBD[26]. In summary, a variety of enteropathies occur with various human health conditions which are governed by a harmful and continuing cycle of gut barrier dysfunction, immune activation, altered gut microbiota, and impaired nutrient absorption[1,13](Figure 1).

Unfortunately, available therapies are often directed at symptoms and not causative factors and include such things as dietary modifications or restrictions, steroids, broad spectrum antibiotics, antidiarrheals, or supportive IV fluids. Due to a broad range of potential causes, multidimensional approaches may be needed, including nutritional interventions alongside current drug treatments to manage these complex disorders. It is well established that plasma-derived protein concentrates (PPC) from bovine, porcine and other sources, when added to the diets of several species of animals, leads to improvements in appetite, weight gain, intestinal growth, and gut barrier function in a number of intestinal disorders[27-30]. Serum-derived bovine immunoglobulin/protein isolate (SBI), specially-formulated to increase IgG and other proteins, has been extensively studied in animal models and recently has been found to be safe and effective in the management of the enteropathy associated with diarrhea-predominant IBS (IBS-D) and HIV infection[31,32]. The purpose of this review is to summarize the scientific evidence supporting the benefits of orally-administered, immunoglobulin-containing protein preparations for host nutrition and protection of gut barrier integrity, particularly as it relates to conditions associated with enteropathy. Studies on the impact of these protein preparations [PPC, bovine serum concentrate (BSC), serum-derived bovine immunoglobulin (SBI)] on host nutrition, gut barrier function, tight junctions, and immune regulation will be summarized.

COMPOSITION OF BOVINE PLASMA- AND SERUM-DERIVED PROTEIN ISOLATES

Plasma-derived protein concentrates are commonly used in animal husbandry to promote growth and modulate

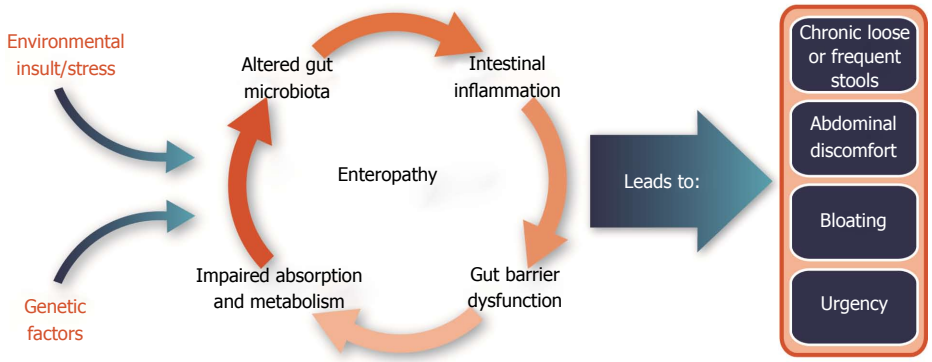

Figure 1 Factors involved in the pathogenesis of enteropathy associated with certain human disease states or conditions (*e.g.*, diarrhea-predominant irritable bowel syndrome or human immunodeficiency virus infections).

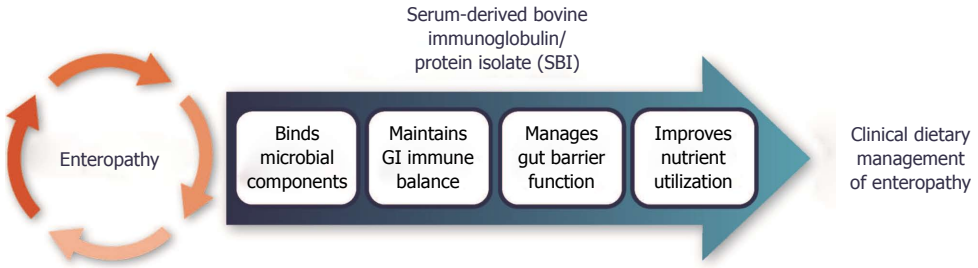

Figure 2 Proposed mode of action for serum-derived bovine immunoglobulin protein isolates to aid management of enteropathy.

intestinal inflammation in immunocompromised young animals[27-30]. Agricultural PPC products are prepared from blood obtained from abattoirs using hygienic collection and processing procedures to ensure high quality plasma products. Preparation first involves the addition of an anticoagulant with subsequent centrifugation to separate the cellular fraction. The plasma is then concentrated by filtration, using inverse osmotic membranes or ultrafiltration and then spray-dried to create a plasma protein powder. During spray drying, plasma proteins are exposed to high temperatures for a very short period of time to avoid denaturation of proteins and to preserve their biological activity[33,34]. In contrast, SBI is produced through a series of shifts in pH and specific salt additions to chilled, edible grade plasma [United States Department of Agriculture (USDA) approved] to reduce the albumin and fibrinogen content and increase the concentration of immunoglobulins and other proteins[35].

Plasma protein concentrates used for animals typically contain over 80% protein on a weight basis, with over 15% of the protein consisting of immunoglobulins (Ig), mainly IgG. In contrast, SBI preparations are specially formulated to increase protein content and reduce levels of albumin and fibrinogen, which results in proportionally higher levels of immunoglobulins. EnteraGam™ is a specially formulated commercial SBI preparation, manufactured according to FDA good manufacturing practice guidelines and intended for human use as a prescription medical food product. It contains about 92% protein (> 50% IgG) with high levels of essential amino acids, including lysine, threonine, tryptophan, and leucine, and is indicated for the clinical dietary management of enteropathy for patients under physician supervision[36]. Arginine and glutamic acid are also found in relatively high levels compared to common dietary sources of protein and the total caloric content of EnteraGam™ is 372 Kcal per 100 g. Although the exact mechanism of how plasma proteins, and SBI in particular, work in patients with chronic loose and frequent loose stools is unknown, findings from both preclinical and clinical studies demonstrate that plasma proteins or SBI has nutritive benefits, binds bacterial endotoxins, supports immune homeostasis, preserves gut barrier function, and promotes a stable microbiota (Figure 2).

NUTRITIONAL BENEFIT

While the purpose of this review is not to provide an extensive summary of all studies conducted on this topic, several representative studies are summarized in Table 1 and the reader is referred to review articles on this topic[27,30]. Torrallardona reviewed the results from 75 trials in 43 publications, to evaluate the feeding and nutritive benefits of PPC from a variety of sources for weaning piglets[30]. Most studies evaluating PPC showed improvements in caloric intake, growth and metabolism, as well as utilization of feed nutrients. Replacement of several high quality protein sources (*e.g.*, meat extracts, soy, pea, potato, skimmed milk, whey, and fishmeal) with PPC at comparable levels led to improved weight gain and feed intake in piglets. Jiang *et al*[37] evaluated growth performance in piglets after pair-feeding a diet containing

Table 1 Weight gain and growth following dietary supplementation with plasma protein concentrates

Animal Model (age)	Impact of dietary supplementation with SBI	Ref.
Piglets: 14-21 d	Superior growth and feed intakes during the first week in 4 of 5 experiments Growth performance improved by the IgG-rich fraction	Pierce *et al*[28]
Piglets: Varying age groups	Consistent improvement in growth, feed intake and sometimes feed conversion; similar results with spray dried plasma from porcine, bovine, and mixed origin	Torrallardona *et al*[30]
Piglets: Weaned at 14 d	Significantly increased mean daily body weight gains and food conversion efficiencies; no difference in protein intake Significantly greater lean body mass and total carcass mass ($P < 0.05$) Significantly lower circulating urea concentrations ($P < 0.05$), indicating greater retention of nitrogen and reduced amino acid catabolism	Jiang *et al*[37]
Piglets: Weaned at 21 d, infected with ETEC K88	Increased average daily weight gain and food intake Protected against *E. coli*-induced inflammation	Bosi *et al*[44]

SBI: Serum-derived immunoglobulin/protein isolates; *E. coli*: *Escherichia coli*; ETEC K88: Enterotoxigenic *E. coli*, K88 strain.

soy protein or PPC for 24 d. Protein intake was similar among groups while the rate of weight gain and protein conversion efficiency was significantly higher in the PPC group, especially during the early weaning period. Pigs fed PPC also had improved body weight and absolute mass of protein with no difference in fat mass, suggesting a higher efficiency of dietary protein utilization for lean tissue growth. Feeding PPC reduced the circulating concentrations of urea, arginine, citrulline and ornithine, suggesting a reduction in the catabolism of amino acids to urea and increased availability of dietary amino acids for lean tissue mass. In addition, there were also significant increases in bone mineral content and bone mineral density in the PPC-fed compared to the soy protein-fed group.

Pierce *et al*[28] conducted several experiments to evaluate the growth and feed intake of weaned piglets fed porcine PPC, bovine PPC, or different molecular weight fractions of PPC. Collectively, the results demonstrated that both porcine and bovine PPC enhanced growth rate and feed intake of weaned piglets, while the IgG fraction of porcine or bovine plasma appeared to stimulate growth performance that was comparable to intact PPC and superior to the albumin or low MW fractions of PPC. These data suggests that a distinct nutritional role may exist for the IgG-rich fraction of PPC to support growth performance.

SAFETY AND DIGESTIBILITY

Plasma-derived protein concentrates (*e.g.*, PPC, SBI) are composed of > 50% IgG and other proteins and peptides that reflect the composition of plasma and are similar to other serum proteins present in colostrum and milk. Such products typically do not contain milk ingredients such as lactose, casein, or whey, so adverse reaction rates would be expected to be minimal. However, patients who have an allergy to beef should not take SBI or PPC products. The rigorous process used to prepare commercial forms of SBI meets strict industry standards to ensure that finished products do not become contaminated with infectious agents, including the bovine spongiform encephalitis (BSE) agent. In addition, SBI has been self-affirmed as Generally Recognized as Safe (GRAS) with no safety-related questions by the US Food and Drug Administration (FDA) for doses up to 50 g/d. SBI has not yet been tested in pregnant or nursing mothers or immunocompromised individuals, so use in such patients should be at the discretion of the patients' physician.

The safety of SBI has been evaluated in both pediatric and adult subjects. Tolerance and digestibility of SBI was evaluated in 12 healthy adult volunteers by Hanning *et al*[38]. Volunteers were administered 10 g of SBI orally and blood samples were obtained at various time points, which showed elevated levels of plasma total amino acids and leucine at 1-2 h following SBI administration. Bovine IgG was not detected in serum samples from study subjects, suggesting that bovine IgG remains in the intestinal tract and does not pass the luminal barrier into the blood stream. Subjects then consumed 5 g of SBI daily for 2 wk and completed daily diaries for general health and adverse events (AEs). No serious AEs were reported by test subjects. The following AEs were reported: increased urination (3); stomach cramps (3); fatigue (2); headache (2); sore throat, softened stools, nausea, constipation, and irritability (1 each). Bovine IgG was detected by enzyme-linked immunosorbent assay (ELISA) in stool samples from test subjects on day 14 but not at baseline (day 0), suggesting survival of some IgG following GI transit, which is similar to previous reports[39,40].

A standard diet with graded amounts of PPC about 0.9 to 2.5 mg/kg BW/d was also fed to infants 9 to 25 mo of age at entry ($n = 10$) recovering from severe protein-energy malnutrition to evaluate acceptability, safety, and digestibility[41]. Study diets were well accepted by study subjects with no evidence of intolerance and no AEs were reported. In another study, malnourished infants (age 6-7 mo of age at entry; $n = 107$) fed a diet containing PPC (about 3.5 g/d) for up to 8 months showed no side effects or adverse impact on growth or morbidity rates when compared to infants fed supplemented with whey protein concentrate[42]. Studies in HIV+ patients ($n = 8$)[31], a longer term open-label exposure in HIV+ patients ($n = 35$) (data on file), and subjects with IBS-D ($n = 66$)[32] also showed only minor or non-medication related adverse events, as well as no clinically relevant

Table 2 Effects of plasma-derived protein concentrates on intestinal function in animal models

Species	Model/indication	Impact of dietary supplementation with SBI	Ref.
Pig	Postweaning	Reduced colonic paracellular permeability Reduced ileal permeability Fewer lamina propria cells in ileum and colon Reduced transepithelial electrical resistance in the colon - improved tight junction Significantly improved fecal scores	Peace *et al*[46]
	Rotavirus infection	Significantly reduced clinical signs of diarrhea Significantly greater intestinal mucosal protein and lactase activity	Corl *et al*[43]
	Infection by ETEC K88	Decreased inflammatory cell infiltration and mucosal damage Increased crypt depth, reduced intestinal expression of proinflammatory TNF-α and IL-8	Bosi *et al*[44]
Rat	Exposure to SEB	Improved ion transport function, as measured by reductions in the potential difference across the jejunum and Na-K-ATPase activity Improved mucosal permeability (dextran flux and HRP paracellular flux)	Pérez-Bosque *et al*[47]

SBI: Serum-derived immunoglobulin/protein isolates; ETEC K88: Enterotoxigenic *Escherichia coli*, K88 strain; TNF-α: Tumor necrosis factor alpha; IL-8: Interleukin 8; SEB: *Staphylococcus aureus* enterotoxin B; Na-K-ATPase: Sodium-potassium adenosine triphosphatase; HRP: Horse radish peroxidase.

changes in blood chemistries or hepatic or renal markers in any studies. Collectively, the results from available clinical studies suggest that SBI is safe and well-tolerated when consumed up to 8 mo in doses ranging from 0.18 to 10 g per day in infants, children and adults.

In order for PPC supplementation to provide benefits to dysfunctional intestinal mucosa, the immunoglobulin and other active protein components must resist digestion and remain active in the lumen of the intestine. Morel *et al*[39] used radial immunodiffusion to evaluate survival of IgG at various points along the intestine in weaned piglets fed PPC. They found 50% undigested IgG located in the proximal small intestine, 17% in mid-small intestine and 10% in the distal small intestine, but none in the cecum and colon. Rodriguez *et al*[40] found IgG survival through the intestinal tract at 8% and 5%, in adult dogs and cats fed PPC or purified IgG, respectively, which suggests partial resistance to digestion. The authors found that the immunoglobulin fraction present in the feces of these animals was the Fab fraction.

IMPACT ON GUT BARRIER AND INTESTINAL RECOVERY

The ability of PPC and SBI to modulate intestinal barrier function, permeability, and malabsorption has been evaluated in a number of preclinical and clinical studies.

Preclinical studies

Studies on the effects of bovine immunoglobulin isolates (PPC or SBI) on inflammation in the GI tract have primarily come from preclinical models in which animals were challenged by infection or exposure to bacterial toxins (Table 2). In one study of piglets infected with rotavirus, PPC was effective at reducing diarrhea, improving intestinal recovery and maintaining growth[43]. Infected soy-fed pigs had significantly greater diarrhea scores ($P < 0.001$) from day 1 to 7 post-infection, while diarrhea scores of infected pigs fed PPC ranked the same as scores from uninfected controls. Administration of PPC was not able to attenuate the reductions in intestinal villus height and the villus height/crypt depth ratio caused by rotavirus infection. Nevertheless, oral feeding of PPC maintained greater intestinal mucosa protein and estimated total lactase activity than infected, soy protein-fed piglets. In a second study, weaned pigs were challenged with enterotoxigenic *Escherichia coli* K88 (ETEC K88), used as a model of *in vivo* pig IBD, to investigate whether PPC could improve growth, immune defense and reduce intestinal inflammation[44]. Compared to a diet based on fish protein, ETEC K88 infected pigs fed PPC showed higher calorie intake and daily weight gain, less intestinal mucosal damage and inflammatory cell infiltration, and reduced expression of pro-inflammatory cytokines.

In a third study of infectious enteritis - *Cryptosporidium parvum* infection in neonatal calves, a disease which produces moderate intestinal inflammation, watery diarrhea, and increased intestinal permeability - Hunt *et al*[45] showed that the daily addition of a bovine serum product (compared with a soy protein control) reduced diarrheal volume, oocyte shedding, and intestinal permeability, while facilitating villus re-growth and increasing mucosal surface area. Lactase activity was significantly improved in response to bovine serum concentrate.

Other data in preclinical models have specifically evaluated tight junction protein expression in response to early weaning and toxin challenge. Peace *et al*[46] evaluated the effects of PPC in piglets undergoing early weaning, a condition known to induce impairment in intestinal epithelial barrier function. Piglets were fed a control diet containing PPC for 7 or 14 d to evaluate impact on ileal and colonic barrier function. Co-administration of PPC with radiolabeled nutrients reduced paracellular permeability as indicated by significant reductions in colonic ^{14}C-inulin permeability on day 7 post-weaning and reduced ileal ^{3}H-mannitol and ^{14}C-inulin permeability on day 14. Protein plasma concentrate also reduced the predominantly lymphocytic cellular infiltration in the lamina propria in both ileum and colon, concomitantly reducing levels of pro-inflammatory cytokines in colon (see below). As shown by immunofluorescence staining, claudin-1, a tight junction protein was more highly expressed and localized to tight

Table 3 Human studies with serum-derived immunoglobulin/protein isolates to evaluate intestinal benefits and quality of life

Species	Model/indication	Impact of dietary supplementation with SBI	Ref.
Human *n* = 8, HIV positive adults	HIV-associated enteropathy	Significant reduction in mean bowel movements/day and improvement in stool consistency scores after 8 wk (P = 0.008) Significant reduction in GI questionnaire scores from 17 at baseline to 8.0 at 8 wk (P =0.008) No change in gut permeability (disaccharide absorption); increase in D-xylose absorption in 7/8 subjects Maintained stool frequency and consistency for an additional 9 mo (n = 5)	Asmuth *et al*[31]
Human *n* = 66 adults	IBS-D	10 g/d showed significant decrease in # symptom days with abdominal pain, flatulence, bloating, loose stools, urgency or any symptom over 6 wk (P < 0.05) 5 g/d showed significant improvements in loose stools, hard stools, flatulence and incomplete evacuation (P < 0.05)	Wilson *et al*[32]
Human *n* = 10 infants or children (9-25 mo)	Malnutrition	Significant reductions in fecal wet and dry weights, and lower fecal fat and energy losses compared with the control diet (P < 0.05) in relation to the amount of SBI in the diet during three randomly ordered 7-d periods	Lembcke *et al*[41]
Human *n* = 259 infants (6-7 mo)	Malnutrition	Trends toward weight gain and upper arm circumference (a measure of lean body mass) increases were found in the SBI + micronutrient group *vs* SBI alone	Bégin *et al*[42]

HIV: Human immunodeficiency virus; IBS-D: Irritable bowel syndrome, diarrhea predominant.

junctions in animals fed PPC.

The protective effects of spray-dried porcine PPC in a rat model of intestinal inflammation were also evaluated[47]. Weaned rats were fed a diet with or without PPC for 14 d then exposed to intraperitoneal challenge with *Staphylococcus aureus* enterotoxin B (SEB) known to disturb barrier function and ion transport. Addition of PPC to diets significantly ameliorated SEB-induced increases in intestinal permeability as measured by dextran flux (P < 0.05) and horseradish peroxidase (HRP) paracellular flux (P < 0.05) across the intestinal epithelium. Plasma protein concentrate was also shown to increase β-catenin expression, part of the adherens complex positioned adjacent to the tight junction. These data suggest that PPC beneficially promoted endogenous repair of the tight junctions, modulated inflammation, reduced permeability, and improved diarrhea in pigs challenged with enterotoxin B.

Collectively, the results of these experimental studies suggest that dietary plasma protein preparations strengthen intestinal barrier function and prevent alterations in intestinal epithelium during inflammation. Two reviews have been published on the effects of PPC and the proteins in SBI on intestinal barrier function in animal models of human disease[48,49].

Clinical studies

Two clinical trials evaluated the efficacy of dietary SBI for improving intestinal absorption, GI symptom scores, and quality of life measures in patients with HIV-associated enteropathy or IBS-D (Table 3). An open-label study was conducted by Asmuth *et al*[31] to evaluate the impact of oral SBI on GI symptoms and systemic markers of immune activation in patients with a diagnosis of HIV-associated enteropathy. To qualify, patients with enteropathy were given an extensive evaluation to exclude other GI disease. Eight patients were enrolled in the study and received 5 g of SBI/d for 8 wk followed by a 4 wk washout period. Administration of SBI led to consistent improvement in GI symptoms associated with HIV enteropathy. After 8 wk of SBI administration, bowel movements per day decreased from 5.8 to 2.0 (P = 0.008) and stool consistency scores (1-formed to 6-watery) improved from 5.3 to 3.0 (P = 0.013). A GI symptom questionnaire showed a marked decrease in score from 6 to 0.5 (P =0.008). After a 4 wk washout period, 5 patients continued on SBI to week 48 maintaining similar bowel movements and stool consistency and GI symptom questionnaire responses. An additional open-label, in-market analysis of 31 patients taking various nutritional formulas which contained 2.5 to 5.0 g SBI showed improved management of loose stools, thus providing further evidence for the management of HIV-associated enteropathy (data on file).

A randomized, double-blind, placebo-controlled study was conducted in individuals with IBS-D to investigate the efficacy of SBI on decreasing gastrointestinal symptom scores and improving the quality of life[32]. Study subjects (n = 66) with a diagnosis of IBS-D for at least 6 mo prior to enrollment met the Rome II diagnostic criteria for IBS. Test groups received placebo (10g /d soy protein isolate), SBI 5 g/d + 5.0 g/d placebo or SBI 10 g/d for 6 wk and completed an IBS-36 questionnaire at baseline (day 0) and at the end of the study (week 6). The daily symptom diary assessed the presence and severity of the following symptoms: nausea, abdominal pain, flatulence, bloating, hard stools, loose stools, urgency, straining, incomplete evacuation and mucus. Forty-five subjects completed the study per protocol and were included in the analysis: 10 g/d SBI (n = 15), 5 g/d SBI (n = 15), and placebo group (n = 13). Results showed that subjects receiving 10 g/d of SBI experienced significant within-group reductions in the number of days with abdominal pain (P < 0.01), loose stools (P < 0.01), bloating (P < 0.05), flatulence (P < 0.01), urgency (P < 0.05) and any symptom (P < 0.01) at EOT *vs* baseline (Table 3). Subjects receiving 5 g/d of SBI (n = 15) reported statistically significant within-group reductions in days with flatulence (P < 0.035), incomplete evacuation (P < 0.05),

Table 4 Effects of serum-derived immunoglobulin/protein isolates administration on immune and inflammatory markers

Species	Model/indication	Impact of dietary supplementation with SBI	Ref.
Pig	ETEC K88	Reduced expression of TNF-α and IL-8 in the gut	Bosi *et al*[44]
	Postweaning	Reduced TNF-α in the colon	Peace *et al*[46]
		Reduced IFNγ levels in the ileum and colon day 7, but not day 14 post weaning	
Rat	SEB	Prevented the SEB-induced increase in IFN-γ, IL-6, and LTB4 in Peyer's patches and in the mucosa	Pérez-Bosque *et al*[69]
		Increased anti-inflammatory cytokines (IL-10 and mature TGF-β) in intestinal mucosa	Pérez-Bosque *et al*[68]
		Reduced SEB-induced increase in cytotoxic lymphocyte populations of γδ-T cells, natural killer cells, and the number of activated T lymphocytes in lamina propria.	
Mouse	Mdr1-/- knockout mouse model of spontaneous colitis	Reduced the percentage of activated Th lymphocytes	Moretó *et al*[48]
		Reduced INF-γ and TNF-α expression in the colon	
		Significantly reduced the expression of cytokines IL-2 and IL-17, chemokines MCP-1 and MIP-1b, and iNOS in the mucosa	
Mouse	2% DSS-induced IBD model	Reduced elevation of IL-1α, IL-4, IL-6, IL-10, MCP-1, and KC	Jiang *et al*[67]
Human (HIV+ adults)	HIV enteropathy	I-FABP fell below baseline in 4/5 patients who continued receiving SBI ($P < 0.12$) out to 48 wk	Asmuth *et al*[31]
		MMP-9/TIMP-1 ratios in subjects were significantly lower than controls at baseline ($P < 0.007$)	
		MCP-1 levels decreased in 5/5 patients who continued receiving SBI ($P < 0.06$) out to 48 wk	

ETEC K88: Enterotoxigenic *Escherichia coli*, K88 strain; TNF-α: Tumor necrosis factor α; IL: Interleukin; SEB: *Staphylococcus aureus* enterotoxin B; IFNγ: Interferon-γ; LTB4: Leukotriene B4; TGF-β: Transforming growth factor β; MCP 1: Monocyte chemotactic protein 1; MIP-1b: Macrophage inflammatory protein; iNOS: Inducible nitric oxide synthase; KC: Keratinocyte-derived cytokine; HIV: Human immunodeficiency virus; I-FABP: Intestinal-fatty acid binding protein; MMP-9: Matrix metalloproteinas-9; TIMP: Tissue inhibitor of metalloproteinase.

and "any symptom" ($P < 0.01$). No significant within group improvements were seen in the placebo group. There were no significant changes in quality of life (QoL) scores or in hematology or clinical chemistry values among the therapy groups.

Studies have also been performed in infants and children recovering from malnutrition. A standard diet with graded amounts of SBI was also administered to infants or children 9 to 25 mo of age at entry ($n = 10$) recovering from severe protein-energy malnutrition to evaluate acceptability, safety, and digestibility during three randomly ordered 7-d dietary periods[41]. Replacing 50% of the protein in the standard diet with SBI led to significant reductions in fecal wet and dry weights, and lower fecal fat and energy losses, suggesting greater absorption of fat and energy compared with the control diet ($P < 0.05$) (Table 3). Investigators suggested that SBI enhanced intestinal recovery from severe malnutrition.

Another randomized, controlled, community-based intervention study evaluated the effects of SBI and/or multiple micronutrients on children's growth, morbidity, and micronutrient status[42]. A total of 259 children who were initially 6 to 7 mo of age received 1 of 4 maize-based dietary products daily for 8 mo with or without protein supplementation. Groups studied: SBI, whey protein concentrate (WPC, control group), SBI plus multiple micronutrients, or WPC plus multiple micronutrients. Two hundred and 25 (225; 86%) children completed ≥ 60 d of observation, 184 (71%) completed ≥ 180 d of observation, and 132 (51%) distributed among the 4 treatment groups finished the full 8 mo of observation. There were no significant differences in growth or morbidity by treatment group for those children who completed 8 mo of observation. Although not statistically significant, there were trends toward weight gain and mid arm circumference (a measure of lean body mass) increases in the SBI+ micronutrient group suggesting better utilization of these nutritional substances (Table 3).

EFFECTS ON GUT MICROBIOTA

Changes in gut microbiota has been identified as one potential factor in causing inflammation that leads to alterations in gut barrier function with associated increases in mucosal permeability. An increase in firmicutes over bacteroidetes bacteria has been reported in IBS patients[50-52]. Another study in IBS-D patients found an increase in bacteroides and clostridia with an associated reduction in bifidobacteria[53]. Pediatric IBS-D patients were reported to show significant differences compared to healthy controls having statistically greater numbers of gammaproteobacteria[54]. There is also a well-recognized dysbiosis that occurs in IBD, although the colonic bacterial imbalances are less well-characterized for ulcerative colitis compared to Crohn's disease[55-58]. Recently it was also reported that the microbiota in both ulcerative colitis and Crohn's disease was relatively unaltered, but metabolism by bacteria in the microbiota was significantly changed[59]. There were notable shifts in fecal metabolome showing reduced carbohydrate processing and alterations in various amino acid biosynthesis pathways. This alteration in gut microbiota may contribute to increased tight junction permeability with associated decreases in barrier function, and changes in bacterial metabolic products and host nutrient malabsorption. Diet may play a role in the causality and/or in the progression of both IBS and IBD[60,61]. Therefore, it is reasonable to assume that diet may play a

role in restoring a natural balance to the gut microbiota and metabolome.

In the HIV-associated enteropathy population treated with SBI, the firmicutes and bacteroidales were the dominant phyla in all 8 patients[62]. When SBI was administered to these patients, proinflammatory gammaproteobacteria decreased from 0.70% to 0.12%. *Clostridium* (genus) decreased from 6.5% to 3.4% in the stool and correlated with duodenal CD3+/CD4+ density (r = -0.63; $P < 0.01$). Ruminococcus and the bacteroidetes/firmicutes ratio, which increased in 6/8 SBI-treated subjects in the study, have been shown to contribute to better calorie utilization from the diet[63,64]. Changes in gut microbiota in the study also correlated with local lymphocyte populations that increased significantly with short-term SBI administration over 8 wk. These results suggest that some component in the formulation may be normalizing gut bacteria, perhaps the IgG fraction. Work is underway to further characterize these interactions.

EFFECTS ON INTESTINAL INFLAMMATION

The release of inflammatory mediators, such as reactive oxygen species, prostaglandins, leukotrienes, and cytokines, from mucosal leukocytes is associated with the altered barrier function and increased permeability caused by intestinal inflammation. Cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α play an important governing role in such inflammatory responses, while other cytokines such as IFN-γ, IL-12 and IL-18 affect the production and cellular response to IL-1β and TNF-α[65]. In models of inflammation where several cytokines are produced, specific blockade of IL-1β and/or TNF-α results in a reduction in the severity of the inflammation[65]. Dietary PPC has been shown to reduce the expression of proinflammatory cytokines and alter the lymphocyte response during immune activation in weaned piglets[46] as well as experimental models of intestinal inflammation in mice[66,67], rats[68-70] and pigs[44] (Table 4). For example, a study by Pérez-Bosque *et al*[71] investigated the effects of dietary SBI on immune responses of mucosal-associated lymphoid tissue in mice with a genetic predisposition to IBD. Wild type (WT) mice and mice lacking the *mdr1a* gene (KO) were fed diets supplemented with either SBI (2% w/w) or milk proteins (control diet) starting on day 19 (weaning). At day 56, SBI reduced the production of the proinflammatory cytokines and chemokines IL-17, IL-6 and CCL4 ($P < 0.05$), prevented the expression of IFN-γ ($P < 0.05$), and blocked the increase in colon crypt permeability that was found in the mdr1a KO model. SBI treatment produced increases in mucosal concentrations of anti-inflammatory TGF-β and in the percentage of regulatory T lymphocytes (both $P < 0.05$), thus reducing the activated Th1 to regulatory Treg lymphocyte ratio. PPC and immunoglobulin-enriched protein isolates have also been demonstrated to affect Peyer's patch lymphocyte populations in weaned rats challenged with *S. aureus* superantigen B (SEB)[70]. In this study, it was shown that the mild intestinal inflammation associated with the SEB model was reduced by dietary PPC as measured by decreased diarrhea. Furthermore, the administration of PPC significantly increased the number of T-helper cells, while reducing the number of activated T-helper cells as compared with animals not fed PPC or immunoglobulin-enriched protein isolates[70]. This same trend was observed for changes in the population of γδ-T cells and natural killer (NK) T cells in the Peyer's patches of rats fed diets containing PPC or immunoglobulin isolates.

In an initial clinical trial in HIV patients with decimated lamina propria CD4+ counts, SBI ingestion significantly increased jejunal CD4+ lymphocyte densities over 8 wk, but had no effect on circulating CD4+ counts[31]. In addition, levels of I-FABP, a marker for enterocyte damage, initially rose in 7/8 subjects after 8 wk, but then fell below baseline in 4/5 who continued taking SBI after 48 additional weeks on product, suggesting that damage to enterocytes caused by inflammation had ceased (Table 4). Collectively, data from these preclinical and clinical studies support the hypothesis that the distinctive protein composition of SBI can play a role in the modulation of the immune response in the intestine.

MECHANISM OF ACTION

SBI contains immunoglobulins, particularly IgG, that are directed against a wide array of pathogens and foreign antigens due to the fact that SBI is prepared from plasma obtained from thousands of animal donors. The Fab regions of IgG recognize antigenic targets and provide diversity to antibodies, while the Fc region interacts with Fc gamma receptors on certain immune cells to enhance phagocytic activity by macrophages, monocytes, and polymorphonuclear neutrophils (PMNs). Several mechanisms may explain how oral immunoglobulins modulate intestinal inflammation and support gut barrier function. For example, immunoglobulins in SBI may simply bind directly to specific microbial pathogens or their toxins, thereby interfering with their ability to migrate through the mucus layer and enter or damage epithelial cells.

A more likely explanation of how oral immunoglobulins work to maintain intestinal homeostasis may involve binding to highly-conserved microbial antigens such as bacterial lipopolysaccharide (LPS), also known as microbe-associated molecular patterns (MAMPs), and interfere with signaling pathways that lead to inflammation (Figure 3). Under normal conditions, cells of the innate immune system play a crucial role in maintaining intestinal homeostasis through a highly-regulated process involving the recognition of MAMPs through pattern recognition receptors (PRRs), such as toll-like receptors (TLRs). TLRs are differentially expressed by various cells of the GI tract (*e.g.*, macrophages, dendritic cells, endothelial cells, myofibroblasts)[72,73] and play a key role in signaling the recognition of MAMPs by activating several

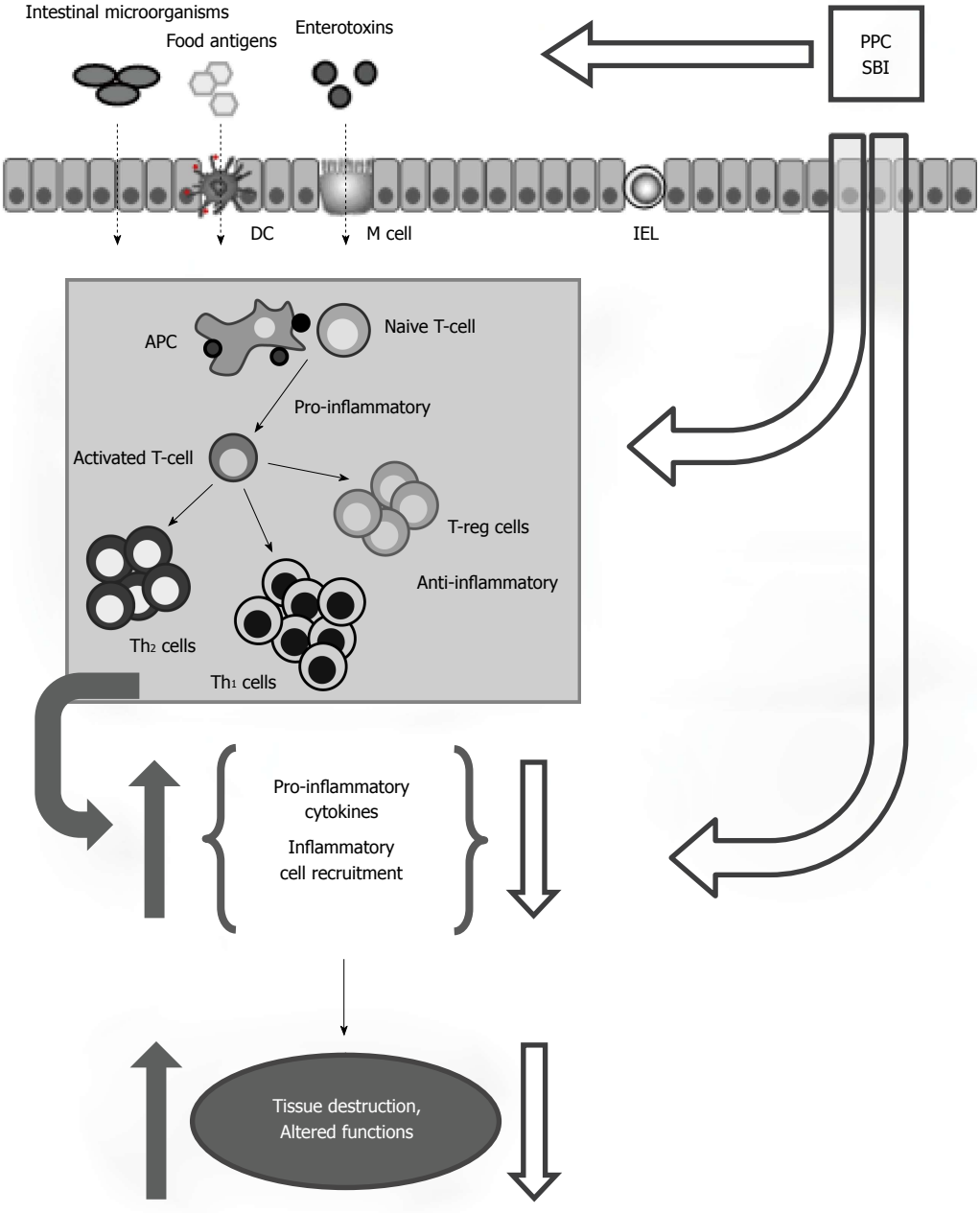

Figure 3 Summary of the postulated mode of action for serum-derived immunoglobulin/protein isolates. Immunoglobulins in SBI support intestinal homeostasis by binding MAMPs (endotoxins, *etc.*), toxins or other antigens in the lumen of the intestinal tract. Immunoglobulin binding interferes with downstream antigen detection by cell surface receptors on IELs or APCs such as DCs and macrophages that influence T cell activation, cytokine production, and barrier fortification. Additionally, biologically active compounds in immunoglobulin isolates may interact directly with mucosal immune cells in the lamina propria to influence mucosal inflammatory responses and epithelial cells to influence barrier function. SBI: Serum-derived immunoglobulin/protein isolates; MAMP: Microbe-associated molecular patterns; IEL: Intraepithelial lymphocytes; APC: Antigen presenting cells; DC: Dendritic cells; T-reg: Regulator T-lymphocytes; Th1: T helper type 1; Th2: T helper type 2. Reprinted with permission from Moretó *et al*[48].

inflammatory pathways, including the NF-κB pathway which is a key regulator of proinflammatory TNF-α, IL-1β, IL-6 and IL-8 cytokine production[74]. Prolonged recognition of MAMPs can lead to a persistent state of inflammation associated with numerous chronic inflammatory disorders such as IBS, IBD, and HIV enteropathy. Studies have shown that the IgG, IgA, and IgM contained in SBI bind to bacterial endotoxins and a wide array of other bacterial, viral, and fungal MAMPs[75,76]. Therefore, it is possible that SBI binding of microbiota components results in less binding of MAMPs by macrophages and dendritic cells which may interfere with release of IL-1, IL-6, and TNF-α[77]. Similarly, less presentation of antigens by dendritic cells and macrophages may result in a decrease in activated T cell populations and more regulatory cell phenotypes that produce IL-10 to dampen inflammation[70].

Alternatively, SBI may contain a large fraction of natural antibodies that work in other ways to maintain immune homeostasis. For example, studies with intravenous immunoglobulin (IVIG) have shown that binding of the Fc portion of IgG to Fc receptors on target cells

may govern some of the anti-inflammatory mechanisms involved with IVIG therapy by up-regulating the expression of inhibitory classes of Fc receptors and down-regulating the activating class of Fc receptors[78]. Autoreactive antibodies in IVIG have also been shown to modulate Th1 and Th2 cytokine production[79], trigger the production of interleukin-1 receptor antagonist, abrogate the capacity of mature dendritic cells to secrete IL-12 upon activation *in vitro*, and enhance anti-inflammatory IL-10 production[80]. Collectively, such immune modulating effects of SBI might explain previous reports of reduced expression of pro-inflammatory cytokines and altered lymphocyte response to immune activation in weaned piglets[46] and experimental models of intestinal inflammation[66-69].

The ability of SBI to modulate inflammation may also benefit the patient with enteropathy by improving gut barrier function. A developing body of evidence indicates that intermittent or even minor inflammation in the intestinal mucosa can elicit changes in intestinal structure and function, leading to increased mucosal permeability[12,81]. For example, increased production of pro-inflammatory cytokines such as TNF-α, IFN-γ, and various interleukins during certain chronic inflammatory disorders[4,82,83] have been shown to increase paracellular permeability by impacting the expression or degradation of claudin and occludin tight junction proteins[84,85]. Conversely, certain anti-inflammatory cytokines such as IL-10 and TGF-β appear to maintain tight junction barrier and protect against intestinal inflammation[82].

In addition to the IgG content of PPC and SBI, the effect on lean body mass may also be in part due to the amino acid content of the complex protein mixture. Plasma protein concentrate and SBI contain amino acids which have been identified to be important for recovery after intestinal damage from infectious agents[43,44,47]. For example, glutamine serves as a preferential energy source for rapidly proliferating immune cells and enterocytes, is a nontoxic transporter of ammonia, and has been linked with maintenance of gut barrier function and cell differentiation[86]. Amino acids absorbed into the blood from PPC or SBI may also play an anabolic role in the body; for example, tryptophan may support the generation of serotonin or metabolite formation in the kynurenine pathway[22-24].

CONCLUSION

Chronic intestinal disorders or enteropathies occur in a variety of human disease conditions such as IBS, IBD, and HIV infection which are characterized by intestinal inflammation, increased gut permeability, and reduced capacity to absorb nutrients. Most therapies used to treat enteropathy are aimed at managing symptoms or target single pathways. However, a multifaceted approach may be needed to manage enteropathy associated with these complicated disease states, or in some cases the side effects of pharmaceutical treatment protocols.

There is a developing body of evidence indicating that intermittent or even relatively minor inflammation can lead to changes in intestinal structure and barrier function[87]. Translocation of bacterial antigens may result in increased production and secretion of pro-inflammatory cytokines, including TNFα, IFN-γ, and interleukins[4,82], which contribute to processes that degrade structural tight junction proteins (*e.g.*, occludins[84], claudins[85]), and contribute to symptoms associated with enteropathy. Inflammation-driven disruption of barrier function has been shown to negatively influence growth in young animals, and also has a range of health consequences in humans[1]. For example, post-infectious IBS is recognized to have inflammatory involvement which may persist months after the initial resolution of infection[88,89] with associated intestinal histological changes and increased intestinal permeability[12,83,90,91]. HIV-associated enteropathy has long been associated with inflammatory damage, decreased barrier function, increased permeability and malabsorption of nutrients[92-94]. Due to increased permeability, microbial translocation markers in HIV patients have been shown to be significant predictors for disease progression and death[95,96]. Serum-derived bovine immunoglobulin/protein isolate may provide a distinctive protein composition to counter intestinal inflammation and the resulting changes in barrier function as well as tight junction permeability to help maintain proper functioning of the intestine. Enteropathy is also associated with chronic undernutrition[97,98]. Malabsorption of nutrients such as bile acids, polyols, fructose, and lactose has been reported to contribute to increased symptoms in patients with IBS[99-101]. A nutritional deficiency of vitamin B6 may also be correlated with IBS symptoms[102]. Nutritional interventions may be needed alongside current drug treatments to effectively manage these complicated disorders.

Results from numerous research studies consistently demonstrate beneficial physiological effects for IgG-containing PPC and SBI protein preparations[27,28,30,33]. SBI contains distinctive nutritional factors that may impart growth and protective benefits by several different mechanisms including binding endotoxin, supporting intestinal barrier function, fostering the growth and maintenance of the normal microbiota, reducing pro-inflammatory cytokine production, and maintaining epithelial tight junctions. In environmentally stressed or disease states, increased cytokine production can promote an increase in enteric epithelial tight junction permeability with resultant antigenic penetration of the gut barrier. These effects may be ameliorated through PPC and SBI preparations *via* the reduction of pro-inflammatory cytokine expression, including TNF-α, IFN-γ, IL-1β, IL-6, IL-8, IL-17, thus facilitating restoration of normal GI function and improved nutritional utilization of accompanying SBI proteins[44,66-70,103].

Safety in humans has been confirmed in five clinical studies in which SBI has been administered. Four clinical trials have reported results that are consistent with the hypothesis that SBI may improve intestinal dysfunction.

The study of SBI in HIV-enteropathy patients[31] in which D-xylose uptake was increased and in infants with malnutrition[41] where fecal wet/dry weights as well as lower fecal fat and energy losses were prevented suggest that this distinct and specially formulated protein mixture is able to restore intestinal structure and functional damage caused by proteolytic enzymes, lymphocytic cytokines, or chemokine-induced damage. Oral SBI may represent a safe and effective option with multiple modes of action to provide for distinctive nutritional requirements in patients with disease-related enteropathy to increase digestion, absorption, metabolism, and utilization of a variety of macro- and micronutrients and facilitate resolution of their gastrointestinal symptoms associated with enteropathy, compared to other protein sources.

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REVIEW

MicroRNAs as novel predictive biomarkers and therapeutic targets in colorectal cancer

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Abstract

Colorectal cancer (CRC) is the third most common cancer in western countries. Despite significant improvement in available treatment options, CRC still remains the second leading cause of cancer-related death. Traditionally, 5-fluorouracil has been used as the main chemotherapy drug for treatment of metastatic CRC (mCRC). However, during the last two decades more effective chemotherapeutic agents such as oxaliplatin, irinotecan and the monoclonal antibodies cetuximab, panitumumab and bevacizumab have been used in clinical practice. More recently, the therapeutic armamentarium has been supplemented by the monoclonal antibodies bevacizumab, cetuximab and panitumumab as well as the protein-trap aflibercept and the small molecule multi-kinase inhibitor regorafenib. One of the major problems for the management of CRC is the inherent or acquired resistance to therapeutic approaches. The discovery of microRNAs (miRNAs), a class of small, endogenous, non-coding, single-stranded RNAs that play a role as post-transcriptional regulators, has added new dimensions to the diagnosis and treatment of cancer. Because miRNAs are important regulators of carcinogenesis, progression, invasion, angiogenesis and metastases in CRC, they might serve as potential predictive and prognostic factors and even as therapeutic targets themselves. Several miRNAs are already known to be dysregulated in CRCs and have been linked to biological processes involved in tumor progression and response to anti-cancer therapies. This review summarizes current therapeutic approaches for treating CRC and highlights the role of miRNAs as novel predictive biomarkers and potential drug targets in CRC patients.

Key words: Colorectal cancer; MicroRNAs; 5-fluorouracil; Epidermal growth factor receptor; Targeted therapy

Core tip: In this review article, we summarize the status quo of the current literature regarding microRNAs and their role in resistance against anti-cancer drugs in colorectal cancer. This Review Article explains how microRNAs influence colorectal cancer, and how these small molecules might be useful as predictive factors and drug targets by themselves.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in western societies with about 1.2 million new cases and 608700 deaths estimated to have occurred in 2008[1]. Outcomes for patients with mCRC still remain poor, with an average survival of less than 30 mo[2]. The majority of CRC cases arise from dysplastic adenomatous polyps. The process of transformation includes a few essential events characterized by the activation of oncogenes such as *KRAS* (Kirsten rat sarcoma viral oncogene homolog), *c-MYC* (v-myc avian myelocytomatosis viral oncogene homolog) and *NRAS* (neuroblastoma RAS viral oncogene homolog) and by inactivation of tumor suppressor genes [*e.g.*, *p53* (tumor protein p53) and *APC* (adenomatous polyposis coli)] or DNA repair genes such as *hMSH2* (human mutS homolog 2) or *hMSLH1*[3]. Although 5-fluorouracil (5-FU) has proven to be moderately effective in CRC as a monotherapy, its combination with the chemotherapeutic drugs oxaliplatin and irinotecan improved the therapeutic outcome. During the last few years, the combination of chemotherapeutic agents with more effective systemic agents such as bevacizumab, panitumumab or cetuximab has been established in clinical practice and improved survival rates in patients with CRC[4,5].

CURRENT COLORECTAL CANCER TREATMENT REGIMENS

5-fluorouracil

5-FU is one of the main chemotherapeutic drugs used for treatment of CRC[6,7]. It exhibits its cytotoxicity by incorporating fluoronucleotides into RNA and DNA molecules, but its main toxic effects are mediated by inhibiting the nucleotide synthetic enzyme thymidylate synthase (TYMS)[7]. However, one of the major problems in managing progressed colorectal cancer is both the inherent and acquired failure of 5-FU-based therapy. Several analyses of 5-FU resistance in CRC have focused on genes involved in pharmacodynamic and pharmacokinetic pathways. One main point has been the activity of TYMS, a key therapeutic target of 5-FU. CRCs that are resistant to 5-FU-based chemotherapy have been shown to possess greater TYMS enzymatic activity than cancers that are sensitive to this therapy[8]. High levels of *TYMS* mRNA or protein expression in liver metastases have also been linked to lack of clinical response to 5-FU *in vivo*[9]. Moreover, a meta-analysis of 24 studies demonstrated that low expression levels of TYMS in metastatic colorectal tumors are associated with greater sensitivity to fluoropyrimidine-based chemotherapy[10]. Additionally, the activity of thymidine phosphorylase (TP), a key enzyme that catalyzes the transformation from 5-FU prodrugs of 5'-deoxy-5-fluorouridine (5'-DFUR) to 5-FU, is closely linked to failure of 5-FU and targeted therapy in CRC cells[11].

EGFR inhibitors

The epidermal growth factor receptor (EGFR) belongs to the ErbB family of receptors that are able to promote tumor cell proliferation in diverse epithelial malignancies[12]. For this reason, EGFR has become an important target in oncology. Monoclonal antibodies against EGFR are used as a standard therapy for some types of solid tumors. The chimeric IgG1 mouse/human antibody cetuximab and the human IgG2 antibody panitumumab are considered to be equally effective in mCRC. However, it has been extensively reported that primary resistance to these agents is mediated by mutations in downstream signaling molecules[13]. Misale *et al*[14] showed that the development of resistance to anti-EGFR treatment in CRC is associated with molecular alterations of *KRAS*. Although KRAS mutations confer strong resistance to anti-EGFR antibodies, not all CRC patients with KRAS wildtype (KRASwt) benefit from these agents. Therefore, there is a need for novel biomarkers to better identify which KRASwt patients would benefit from this therapy[15]. A few studies have shown that high EGFR gene copy number could be a potential favorable marker for anti-EGFR therapy in CRC, as patients with low gene copy number are unlikely to respond to anti-EGFR agents[16,17]. Very recently, rare mutations in exons 3 and 4 in the both the *KRAS* gene and *NRAS* gene have demonstrated predictive potential in regards to panitumumab treatment[18].

VEGF targeted therapy

Angiogenesis is widely regarded as an important therapeutic target in many different types of cancer, including CRC[19]. It has been suggested that inhibition of angiogenesis in tumors can influence the development of new tumor blood vessels and probably lead to normalization of the existing tumor vasculature. The vascular endothelial growth factor (VEGF) is a pro-angiogenic factor that plays a key role in tumor vascular development[20]. VEGF-neutralizing antibodies can prevent the binding to and activation of VEGFR1 (vascular endothelial growth factor receptor 1), VEGFR2 (vascular endothelial growth factor receptor 2) and the co-receptors NP1 (Neuropilin 1) and NP2 (Neuropilin 2). Bevacizumab, a humanized monoclonal antibody that binds to all isoforms of VEGF, is the first anti-angiogenic therapy approved for treatment of CRC[21,22]. It has also been used in combination with common chemotherapeutic agents, such as 5-FU and capecitabine as well as irinotecan and oxaliplatin[23]. Resistance to combinatorial therapy in CRC treatment may be due to resistance to bevacizumab, resistance to the chemotherapeutic with which bevacizumab was administered or a resistance to both. As observed, several pathways in addition to the VEGF pathway are involved in angiogenesis. Therefore, mechanisms of resistance to anti-angiogenic therapy may also include VEGF-independent anti-angiogenesis pathways[24].

MIRNA BIOGENESIS AND FUNCTION

MiRNAs are a class of small, endogenous, non-coding, single-stranded RNAs that play a role as post-transcriptional regulators by suppressing the translation or inducing the mRNA degradation of their target genes[25]. Dysregulated miRNA expression in human cancers plays a role in carcinogenesis mainly by regulating their mRNA targets, which could be oncogenes or tumor suppressors[26]. Various combinations of miRNAs are expressed in different cell types and regulate cell-specific target genes. MiRNAs regulate about one-third of all human genes and a single miRNA can target around 200 or more transcripts that are key regulators of multiple signaling pathways in the cell[27,28]. Aberrational miRNA expression patterns, commonly seen in human diseases including various types of cancer, can serve as prognostic factors and may have implications for cancer stem cell regulation[29-32]. More than 50% of known human miRNA genes are localized in fragile chromosomal regions that are susceptible to amplification, deletion or translocation during cancer development[33].

MiRNAs are transcribed as long primary transcripts called pri-miRNAs and are processed into precursor miRNAs (pre-miRNAs) in the nucleus by the enzyme Drosha. These short hairpin RNAs of approximately 70 nt are then translocated to the cytoplasm where they are cleaved by the enzyme Dicer to generate the miRNA duplex[34,35]. Afterwards, the miRNA is unwound by a helicase and one of the strands is defined as the mature strand while the other one is quickly degraded. The mature miRNA is incorporated into an RNA-induced silencing complex (RISC) that mediates gene silencing[36].

MiRNAs are important regulators of oncogenesis, progression, invasion, angiogenesis and metastasis in colorectal cancer. Both upregulation and downregulation have been linked to carcinogenesis in CRC[37]. Many proteins that play a role in key signaling pathways of CRC seem to be influenced by miRNA regulation, such as members of the Wnt/beta-catenin and phosphatidylinositol-3-kinase (PI-3-K) pathways, KRAS, p53, extracellular matrix regulators as well as epithelial-mesenchymal transition (EMT) proteins and transcription factors[26,38,39].

New findings suggest that miRNAs could be linked to sensitivity to chemotherapeutic drugs in tumor cells. For this reason, researchers are interested in the potential role of miRNAs in pharmacogenomics[40]. A recent study by Pardini *et al*[41] provided evidence that a modulation of the expression of base excision repair (BER) genes, such as a post-transcriptional change caused by microRNAs, could influence the efficiency of this repair system. Single-nucleotide polymorphisms (SNP) within the 3'-untranslated regions (UTR) of target genes could lead to an alteration in binding of specific miRNAs that modulate gene expression. Such changes could affect cancer prognosis and therapy outcomes. Hence, characterization of polymorphisms in miRNA-related genes or target sites might afford a basis for miRNA-based therapy approaches.

In the next few pages, we will discuss particular miRNAs which have been experimentally proven to play a role in drug resistance in the past few years.

MIRNAS AND THEIR INVOLVEMENT IN RESISTANCE TO ANTI-CANCER THERAPIES

Let-7

Clinical trials have demonstrated that KRAS mutations are negative predictive biomarkers for EGFR-targeted therapy in CRC. Mechanisms of post-transcriptional downregulation of mutated KRAS might therefore be of clinical relevance in patients with mCRC[42,43]. The members of the *let-7* family are known to target KRAS and their involvement in response to EGFR-targeted therapy has already been reported[44]. Upregulation of *let-7* expression levels might provide a survival advantage by inhibiting mutated KRAS under EGFR-targeted therapy. In addition, *let-7* may reveal further favorable effects by regulating other genes such as the cell cycle regulators, *Myc, Bcl-2* (B-cell CLL/lymphoma 2), integrins and signal transducers. Ruzzo *et al*[45] analyzed the expression levels of *let-7a* in colorectal carcinomas with mutated *KRAS* and in mCRC patients treated with cetuximab and irinotecan. Their study revealed that intra-tumor expression of *let-7a* correlates with tumor response and overall survival in mCRC patients treated with anti-EGFR-based therapy in both *KRAS* mutant and wildtype populations. Moreover, they showed that downregulation of *let-7e* and *let-7b* can potentially be used to predict resistance to the monoclonal antibody cetuximab. Cappuzzo *et al*[46] investigated whether microRNAs can predict sensitivity to EGFR-targeting monoclonal antibodies in patients with mCRC. They identified the cluster *Let-7c/miR-99a/miR-125b* as a signature linked to an outcome different from that of EGFR targeting therapies. In the first cohort, patients with high-intensity signatures showed a significantly longer progression-free survival and longer overall survival. Moreover, in the KRAS wild-type population, high-intensity signature patients had a significantly longer progression-free survival, as demonstrated in the validation cohort. Therefore, the *miR-99a/let-7c/miR-125b* signature could improve the selection of patients with KRAS wild-type mCRC for treatment with EGFR targeting therapies.

Salendo *et al*[47] performed a genome-wide miRNA profiling in 12 colorectal cancer cell lines and established an individual in vitro signature for chemoradiosensitivity. Their study demonstrated that high expression of *let-7g* was linked with a good prognosis in rectal cancer patients. This finding suggests that *let7g* expression may serve as potential predictive biomarker.

MiR-126

An increasing number of studies proposed *miR-126*

as a player in the regulation of angiogenesis, a process that has already been considered as a target for development of novel drugs. High expression of *miR-126* has already been associated with increased vascular endothelial growth factor A (VEGF-A) mediated signaling in endothelial cells and a higher blood vessel integrity[48]. Additionally, *miR-126* has been identified as a putative tumor suppressor in primary tumors[49-51]. Hansen *et al*[48] investigated the role of miR-126 as a predictive marker in patients with CRC in relation to first-line treatment with capecitabine, a precursor of 5-FU, and oxaliplatin (XELOX). They demonstrated a significant relationship between expression of *miR-126* in the primary tumor and sensitivity to first-line XELOX treatment. Low expression of *miR-126* might therefore lead to tumor vessels with decreased integrity followed by an increase in interstitial pressure, which may explain the lower sensitivity in patients treated with XELOX. A recent study by Hansen *et al*[52] revealed that high expression of *miR-126* in mCRC patients was strongly related to a longer progression-free survival. Their results confirm their previous findings on the prognostic value of *miR-126* in mCRC. As VEGF-A may be a target of *miR-126*, the results of their study might provide predictive information in regards to next-generation anti-angiogenic therapy approaches.

MiR-31

Several studies revealed that *miR-31* is upregulated in CRC, but there is little known about its role in modulating tumor cell response to chemotherapeutic drugs. Wang *et al*[53] showed that the treatment of HCT-116 colon cancer cells with an anti-*miR-31* inhibitor increased the sensitivity to 5-FU as early as 24 hours after exposure without affecting cell proliferation. Combination of 5-FU treatment with a respective negative control did not lead to a reduction in cell viability. The apoptosis rate of HCT-116 cells treated with both 5-FU and the anti-*miR-31* inhibitor was the highest among respective control groups, indicating that these agents inhibited proliferation *via* the apoptotic mechanism.

MiR-192/miR-215

The expression levels of *miR-192* and *-215* were shown to be significantly reduced in CRC tissues compared to non-tumor counterparts. Furthermore, Chiang *et al*[54] demonstrated that low expression levels of *miR-192* and *-215* are related to increased tumor size in CRC. Thus, *miR-129* and *miR-215* could be useful biomarkers in the carcinogenesis of CRC. In addition, *miR-192* and *miR-215* were reported to negatively regulate CRC cell proliferation[55]. Boni *et al*[40] showed that miR-192 and -215 downregulate TYMS expression and thus increase resistance to 5-FU in CRC cell lines. TYMS plays a role in normal cell function and is a potential target for chemotherapeutic drugs such as 5-FU. Transcriptional and translational regulation of TYMS most likely affect cell chemosensitivity. Additionally, they demonstrated that *miR-192* and miR-*215* inhibit progression into the S phase, play a role in cell cycle control and prevent sensitivity to 5-FU. Zhang *et al*[56] identified a set of 6 miRNAs including miR-215 which could serve as an authentic prognostic and predictive tool for determination of disease recurrence in patients with stage Ⅱ colon cancer. miR-215 could potentially predict which patients would benefit from adjuvant chemotherapy, which can in turn facilitate patient consultation and help individualize management of patients with this disease.

MiR-148a

It has been shown that *miR-148a* is a pro-apoptotic miRNA in CRC which acts by targeting *Bcl-2*, a regulator of apoptosis. In addition, several in vitro studies have demonstrated that *miR-148a* functions as a tumor suppressor by targeting several genes such as *PXR* (nuclear receptor subfamily 1, group I , member 2), *TGIF2* (TGFB-induced factor homeobox 2), *MSX1* (msh homeobox 1), *CDC25B* (cell division cycle 25B), *DNMT1* [DNA (cytosine-5-methyltransferase 1)] and *DNMT3b* [DNA (cytosine-5-methyltransferase 3 beta)][57]. Takahashi *et al*[58] showed that low expression of *miR-148a* is significantly linked to an unfavorable outcome in treatment of stage Ⅲ CRC patients with 5-FU. They also demonstrated the link between decreased miR-148a expression and poorer sensitivity and survival rate in patients with stage Ⅳ CRC treated with oxaliplatin combined with 5-FU. Stage Ⅳ CRC patients that showed a high *miR-148a* expression level were shown to benefit from chemotherapeutic drugs, indicating that *miR-148a* may have predictive value in the assessment of response to CRC treatment. In addition, their data suggested that downregulation of *miR-148a* is mediated by DNA methylation. Takahashi *et al*[58] showed that there is a significant and independent association between *miR-148a* methylation and poor survival in stage Ⅳ patients. Therefore, the methylation status of *miR-148a* might be a potential prognostic marker in CRC. Kjersem *et al*[59] investigated microRNAs in plasma as potential predictive markers for sensitivity to oxaliplatin-based chemotherapy. Their study revealed that a high expression level of *miR-148a* is associated with a decrease in progression-free survival. The results of their study suggest that *miR-148* could serve as a non-invasive biomarker for predicting outcomes in mCRC patients treated with 5-FU and oxaliplatin-based chemotherapy.

MiR-21

Upregulated *miR-21* expression is associated with some types of human cancer, including CRC. It has been demonstrated that miR-21 regulates several tumor suppressors such as p21, TGF-beta receptor Ⅱ and B-cell leukemia/lymphoma 2-associated X protein. Moreover, overexpression of *miR-21* is linked to poor response to 5-FU-based chemotherapy. Valeri *et al*[60] found an inverse expressional correlation of miR-21 and the tumor suppressor hMSH2. They demonstrated that *miR-21* directly targets the 3' UTR of *hMSH2* mRNA and significantly reduces its protein expression. CRC cells that showed high expression levels of *miR-21* have decreased hMSH2

protein expression and revealed significantly reduced 5-FU-induced G2/M damage arrest and apoptosis. This indicates a characteristic defect in the core mismatch repair system, suggesting that *miR-21* might act as a regulator of genes associated with cell-cycle arrest and/or drug resistance. In addition, Deng *et al*[61] showed that forced miR-21 expression in HT-29 colon cancer cells significantly inhibited apoptosis, enhanced cell proliferation and invasion and increased resistance to the chemotherapeutic agent 5-FU. Moreover, they demonstrated that silencing of *miR-21* inverted these effects on HT-29 cells and restored the sensitivity to 5-FU.

MiR-129

Reduced *miR-129* expression levels have been reported in several tumor cell lines and primary tumors including CRC[62]. Karaayvaz *et al*[63] demonstrated that *miR-129* expression was clearly lower in CRC patients. There was no significant difference between adenoma and stage I and Ⅱ carcinomas. They observed that *miR-129* expression was dramatically reduced in stage Ⅲ and Ⅳ cancers. Hence, their results suggested that loss of *miR-129* is linked to progression in CRC. Additionally, they identified *miR-129* as a novel regulator of *Bcl-2* expression. The expression of *miR-129* promoted apoptosis, inhibited cell proliferation and caused cell-cycle arrest in CRC cells. *miR-129*-based therapies could help to achieve a multi-targeted anticancer therapeutic strategy. 5-FU-based chemotherapy still remains the main option for advanced mCRC treatment, but the possibility of miR-129 restoration may lead to a new strategy in reducing resistance to chemotherapeutic drugs. Furthermore, Karaayvaz *et al*[63] showed that *miR-129* is a suppressor of the 5-FU target protein TYMS and therefore enhances chemosensitivity to 5-FU. In their *in vivo* tumor xenografts, they demonstrated that increasing miR-129 expression to normal levels using synthetic miRNAs made tumors more sensitive to 5-FU-based drugs.

MiR-19b

MiR-19b is one of the 6 miRNAs which are encoded by the *miR-17-92* cluster. The activation of this cluster is mediated by the transcription factor E2F1 which accumulates early in the G1 phase of the cell cycle, suggesting that miRNAs generated from this cluster might play a role in the G1 phase. Kurokawa and collegues found that *miR-19b* expression is upregulated in the DLD-1/R colon cancer cell line, but they did not observe any changes in the cell cycle profile after 5-FU treatment. In addition, they demonstrated that *miR-19b* expression correlated with response to the widely-used anti-cancer drug 5-FU[64]. Likewise, a recent study showed that *miR-19a*, a paralogue of *miR-19b*, is upregulated in HCT-119 and HT29 cells in response to 5-FU-based treatment[65].

MiR-34a

MiR-34a is a member of the *miR-34* family which also includes *miR-34b* and *miR-34c*. Low expression levels of *miR-34a* have been identified in various types of tumors, including CRC[66]. Moreover, *miR-34a* was observed as one of the most downregulated miRNAs in the 5-FU-resistant DLD-1 CRC cell line. Akao *et al*[67] showed that exposure to 5-FU activated the PI3K/Akt signaling pathway in the resistant DLD-1 cell line in comparison to the parental cells and led to an apparent increase in growth. In addition, *miR-34a* expression in the 5-FU-resistant cell line was prolonged at a low level, whereas it showed an upregulation in the parental cells after treatment with a 5-FU-based drug. They observed an upregulation of Sirt-1, a target gene of *miR-34a* which is associated with drug resistance, in the 5-FU-resistant cells and also that silencing of Sirt-1 significantly increased the sensitivity to 5-FU in the 5-FU-resistant cells. This suggested that *miR-34a* is a negative regulator of 5-FU resistance in the CRC cell line DLD-1.

MiR-143

A few studies showed that *miR-143* expression is low in tumors compared to their normal counterparts, both at adenomatous and cancer stages of colorectal neoplasia, as well as in CRC cell lines[68]. In addition, *miR-143* has been identified to directly target the *KRAS* mRNA[69]. Borralho *et al*[70] investigated the role of *miR-143* in the HCT116 CRC cell line. They showed that transient overexpression of *miR-143* led to a 60% reduction in cell viability and stable overexpression of *miR-143* resulted in decreased viability and increased cell death after treatment with 5-FU. These changes were linked to increased nuclear fragmentation and caspase -3, -8 and -9 activities. Furthermore, they demonstrated that exposure of *miR-143*-overexpressing cells to 5-FU resulted in downregulation of the extracellular-regulated protein kinase 5 and Bcl-2 protein expression. In addition, *miR-143* led to increased sensitivity to 5-FU- based drugs, suggesting that *miR-143* is involved in CRC development and plays a role as a chemosensitizer to 5-FU.

MiR-203

Zhou *et al*[71] recently showed that *miR-203* is upregulated in three oxaliplatin-resistant CRC cell lines. They demonstrated that exogenous expression of *miR-203* in chemonaïve CRC cells reduced sensitivity of cells to oxaliplatin treatment. Silencing of *miR-203* expression led to increased sensitivity of CRC cells to oxaliplatin. Furthermore, they performed an *in-silico* analysis and identified ataxia telangiectasia mutated (*ATM*), a primary mediator of the DNA damage response, as a potential target of *miR-203*. Their study showed that mutation of the putative *miR-203* target region in the 3' untranslated region of *ATM* mRNA eliminated the inhibitory effect of *miR-203* on *ATM*. In addition, they demonstrated that stable knockdown of *ATM* led to resistance to oxaliplatin in chemo-naïve CRC cells.

MiR-200c

Hur *et al*[72] reported that *miR-200c* expression modulates EMT in colorectal metastasis. In their study, they demonstrated for the first time that the *miR-200c/429*

cluster was significantly overexpressed in liver metastasis in comparison to primary CRC and that the expression of these miRNAs was specifically regulated by aberrant methylation of their promoter regions. A recent study by Toiyama *et al*[73] revealed that the serum levels of *miR-200c* might serve as potential prognostic and metastatic-predictive biomarkers in CRC patients. They showed that *miR-200c* expression in serum is strongly associated with a metastatic phenotype in CRC; in particular, expression of *miR-200c* in serum was a good predictive marker for lymph node metastasis. Moreover, they demonstrated that *miR-200c* expression in serum can be used as a prognostic and predictive marker of tumor recurrence in patients undergoing curative surgery.

CONCLUSION

Currently, therapeutic agents including 5-FU, oxaliplatin, irinotecan, bevacizumab cetuximab, panitumumab, aflibercept and regorafenib are widely used in clinical practice for CRC treatment. However, many patients are resistant or develop secondary resistance to these agents, highlighting the necessity for the development of novel prognostic markers for drug resistance. As important regulators of gene expression, miRNAs possess high potential as predictive markers for therapeutic response to chemotherapeutic drugs. On the other hand, targeting miRNAs that are involved in the resistance mechanism may improve the therapeutic efficacy in chemo-resistant patients. We foresee that in the near future, miRNA-based prognostic tools could be developed to aid in patient selection for certain treatments, and miRNA-based therapeutics may finally reach the clinical stages.

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Impact of *Clostridium difficile* infection on inflammatory bowel disease outcome: A review

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Abstract

Although a considerable number of studies support a substantial increase in incidence, severity, and healthcare costs for *Clostridium difficile* infection (CDI) in inflammatory bowel disease (IBD), only few evaluate its impact on IBD outcome. Medline and several other electronic databases from January 1993 to October 2013 were searched in order to identify potentially relevant literature. Most of the studies showed that IBD patients with CDI present a greater proportion of worse outcomes than those without CDI. These patients have longer length of hospital stay, higher rates of colectomies, and increased mortality. Patients with ulcerative colitis are more susceptible to CDI and have more severe outcomes than those with Crohn's disease. However, studies reported variable results in both short- and long-term outcomes. Contrasting results were also found between studies using nationwide data and those reporting from single-center, or between some North-American and European studies. An important limitation of all studies analyzed was their retrospective design. Due to contrasting data often provided by retrospective studies, further prospective multi-center studies are necessary to evaluate CDI impact on IBD outcome. Until then, a rapid diagnosis and adequate therapy of infection are of paramount importance to improve IBD patients' outcome. The aim of this article is to provide up to date information regarding CDI impact on outcome in IBD patients.

Key words: *Clostridium difficile* infection; Ulcerative colitis; Crohn's disease; Outcome

Core tip: This review summarizes the impact of *Clostridium difficile* infection (CDI) on inflammatory bowel disease (IBD) outcome. Most of the studies showed that IBD patients with CDI have more of the whole range of short- and long-term worst outcomes than those without CDI. Patients with ulcerative colitis have more severe outcomes than those with Crohn's disease. A prompt diagnosis and adequate treatment of CDI are of paramount importance to improve IBD patients' outcome.

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INTRODUCTION

Over the past 15 years, both incidence and severity of

Clostridium difficile (*C. difficile*) infection (CDI) have increased dramatically worldwide[1,2]. In addition to broad-spectrum antibiotic therapy[3,4], other potential risk factors such as advanced age, prolonged hospitalization, immunosuppression, multiple co-morbidities, the use of proton pomp inhibitors, and the occurrence of a hypervirulent strain of *C. difficile* known as NAP1 (North American pulso-type 1) in some North-American and European areas, have been identified[5-10].

Referring to the same period, several studies clearly demonstrated a significant increase in CDI incidence in patients with inflammatory bowel diseases (IBD)[11-17]. Both ulcerative colitis (UC) and Crohn's disease (CD) present high-risk for CDI, although patients with UC are more susceptible than those with CD[11,12,15,16]. Overall, IBD patients with CDI show more of the whole range of short- and long-term worst outcomes than those without CDI or with CDI alone[11-13,16-19]. However, studies report variable results concerning mortality and colectomy rates, length of hospital stay, and healthcare costs for IBD patients with CDI[11,13,14,16,18-21].

This review aims to summarize available literature regarding CDI impact on both short- and long-term outcome in adult IBD patients.

RESEARCH

A systematic literature search was performed on Medline/PubMed, EMBASE, Scopus, Science Direct, CINAHL, and Web of Science (ISI Web of Knowledge) databases from January 1993 to October 2013 using various combinations of the following key words: "inflammatory bowel disease", "ulcerative colitis", "Crohn' s disease" and "*Clostridium difficile* infection", "*Clostridium difficile*-associated diarrhea", "pseudomembranous colitis". We included only English written studies carried out on adults, from all geographic regions. A manual search of references from the identified studies was also undertaken to identify any additional studies that may have been missed in the computed-assisted literature search. As our objective was to assess the impact of CDI on IBD patients' outcome, only studies reporting outcome of IBD patients co-infected with CDI were taken into analysis. The following data were extracted from each study included: length of hospital stay, colectomy rate, mortality, and healthcare costs. In addition, given the increased need for surgical intervention in UC patients with CDI, a short review of *Clostridium difficile* enteritis and pouchitis has also been made.

SHORT-TERM OUTCOMES

Length of hospital stay

Studies report different results concerning the length of hospital stay in IBD patients with CDI: some report similar stays[12,19], some shorter ones[14], while others (Table 1)[11,13,16,17,21] refer to longer stays than in patients without CDI or with CDI alone. Jodorkovsky *et al*[19] reported a similar mean length of hospital stay in days for IBD (UC) patients with superimposed CDI and those without CDI (11.7 *vs* 11.0, $P = 0.70$), while Bossuyt *et al*[14] found significantly shorter stays in IBD patients with CDI (mean 15.2 d) as compared to non-IBD patients co-infected with *C. difficile* (mean 27.7 d) ($P < 0.001$). By contrast, other studies reported longer length of hospitalization in IBD patients with CDI than in those with IBD alone[11,13,16,17,21]. Thus, Issa *et al*[11], in a retrospective, observational study evaluating IBD patients followed in the Inflammatory Bowel Disease Center, Medical College of Wisconsin, Milwaukee, United States, found a mean length of hospital stay of 13.5 d for their UC patients admitted with CDI as compared to 6 d for those without CDI. From the same center, in a study using nationwide data (including over 2000 IBD patients with CDI, over 44000 with CDI alone, and more than 77000 with IBD alone) was found a 3-d longer hospital stay in IBD patients with CDI. Nguyen *et al*[16] using the Nationwide Inpatient Sample (NIS) reported a 65% increase in the number of days for CD and 46% for UC patients with CDI as compared to non-IBD patients. A recent study using Hospital Episodes Statistics (HES) which covers all in-patient activity delivered by NHS hospitals in England, reported a 27.9 d longer hospital stay in patients with IBD complicated by hospital-acquired CDI than in those with IBD alone[17], much higher than in the above two mentioned North-American studies using similar national datasets[13,16], the difference being partially accounted for by data collection methods. From Canada, a retrospective population-based cohort study of 181 UC patients with CDI and 1835 without CDI hospitalized in Ontario, between 2002-2008, reported a significantly increased mean length of hospital stay in UC patients co-infected with *C. difficile* (11 d *vs* 6 d, $P = 0.0001$)[21].

Colectomy rate

Contrasting results have also been reported regarding colectomy rate in IBD patients with superimposed CDI. Analysis of the NIS HCUP (Healthcare Cost and Utilization Project) data containing more than 90% of United States community hospital discharges[22], showed a six fold (OR = 6.6; 95%CI: 4.7-9.3) increase in colectomy rate in IBD patients with concomitant CDI in comparison with CDI patients without underlying IBD[13]. It should be underlined that this analysis also includes IBD patients admitted for elective surgery, a fact which contributed to such a high colectomy rate[23]. However, other studies too reported higher colectomy rates in IBD patients with CDI than in CDI-free IBD population or in patients with CDI alone[11,17-19]. One case-control study reported a 23.4% emergent colectomy rate in patients with both CDI and IBD (UC) as compared to 13.5% (OR = 2.09, 95%CI: 0.72-6.1; $P = 0.17$) in those with IBD alone[19]. Another study reported a rate of urgent colectomy as high as 45% in hospitalized patients with IBD colitis and co-existing CDI in 2004, which decreased to 25% in 2005, probably due to changes in the treatment

Table 1 Main short-term outcomes in inflammatory bowel disease patients with *Clostridium difficile* infection as compared to those with inflammatory bowel disease alone or *Clostridium difficile* infection alone

Ref.	Journal and year of publication	Study design and time frame	Outcome
Murthy *et al*[21] Canada	Aliment Pharmacol Ther 2012	In-patients, Ontario, Canada, 2002-2008	Increased LOS (11 d *vs* 6 d, $P = 0.0001$), similar rate of colectomy (12% *vs* 9.8%; $P = 0.30$), and higher mortality rate (3.3% *vs* 0.38%, $P < 0.0001$) as compared with UC patients without CDI
Navaneethan *et al*[24] United States	J Crohns Colitis 2012	Out-/in-patients; 2002-2007	No significant difference in the colectomy risk within 3 months of index admission between UC patients with CDI and those with UC alone
Ananthakrishnan *et al*[28] United States	Aliment Pharmacol Ther 2012	In-patients; 1998-2010	4.4% colectomy and 15.2% mortality rates
Jen *et al*[17] United Kingdom	Aliment Pharmacol Ther 2011	Case-control analysis of United Kingdom Hospital Episodes Statistics, out-/in-patients; 2002-2007	Increased mortality (OR = 6.32), higher risk for surgery (OR = 1.87), and 27.9 d longer LOS than patients with IBD alone
Ananthakrishnan *et al*[18] United States	Inflamm Bowel Dis 2011	Case-control analysis of NIS database, out-/in-patients; 1998, 2004, 2007	Increase in colectomy rate from 1998 (OR = 1.39, 95%CI: 0.81-2.37) to 2007 (OR = 2.51, 95%CI: 1.90-3.34) ($P = 0.03$), and in mortality risk (1998: OR = 2.38, 95%CI: 1.52-3.72) (2007, OR = 3.38, 95%CI: 2.66-4.29) ($P = 0.15$)
Kaneko *et al*[25] Japan	Clin Res Hepatol Gastroenterol 2011	Out-/in-patients; 2006-2009	No association between CDI and colectomy rate in UC patients
Kariv *et al*[20] United States	J Crohns Colitis 2011	Out-/in- patients with UC; 2000-2006	No difference in colectomy rates (48% *vs* 50.9%, $P = 0.81$) between infected and non-infected UC patients, no mortality in UC patients with or without CDI
Jodorkovsky *et al*[19] United States	Dig Dis Sci 2010	In-patients; 2004/06-2005/06	Similar mean LOS for IBD patients with CDI and those without CDI (11.7 d *vs* 11.0 d; $P = 0.70$); similar use of cyclosporine therapy (48% *vs* 47%); higher emergent colectomy rate (23% *vs* 13.4%, $P = 0.17$)
Bossuyt *et al*[14] Belgium	J Crohns Colitis 2009	In-patients; 2000-2008	LOS shorter as compared to non-IBD patients (15.2 d *vs* 27.7 d, $P = 0.001$); one patient with UC+ CDI had a semi-urgent colectomy; no mortality in IBD patients, 2 deaths in non-IBD patients
Ricciardi *et al*[15] United States	Dis Colon Rectum 2009	Case-control analysis of NIS database, out-/in-patients; 1993-2003	Increased case fatality in UC+CDI patients but not in those with CD+CDI; operative mortality for UC+CDI patients reached 25.7%
Ben-Horin *et al*[26] Israel and some European countries	Clin Gastroenterol Hepatol 2009	Multi-center, in-patients; 2000-2008	Low colectomy rate (6%) in IBD patients with CDI
Ananthakrishnan *et al*[13] United States	Gut 2008	Case-control analysis of NIS database, out-/in-patients; 2003	Four-fold higher mortality rate (OR = 4.7, 95%CI: 2.9-7.9) compared with IBD alone and twice higher than in those with CDI alone (OR = 2.21, 95%CI: 1.4-3.4); 3-d longer compared with IBD alone; six-fold greater risk of bowel surgery than those with CDI alone (OR = 6.6, 95%CI: 4.7-9.3); 11406 higher hospital adjusted charges
Nguyen *et al*[16] United States	Am J Gastroenterol 2008	Case-control analysis of NIS database, out-/in-patients; 1998-2004	Increased mortality in UC (OR = 3.79, 95%CI: 2.84-5.06) but not in CD patients (OR = 1.66, 95%CI: 0.75-3.66); increased LOS with 65% for CD and 46% for UC, and increased hospital charges compared with non-IBD patients
Rodemann *et al*[12] United States	Clin Gastroenterol Hepatol 2007	In-patients, 1998-2004	LOS similar to non-IBD patients
Issa *et al*[11] United States	Clin Gastroenterol Hepatol 2007	Observational study, out-/in-patients; 2004-2005	Increased LOS (13 d *vs* 6 d); the colectomy rate in UC+CDI decreased from 45% in 2004 to 25% in 2005

C. difficile: *Clostridium difficile*; CDI: *Clostridium difficile* infection; CD: Crohn's disease; IBD: Inflammatory bowel disease; LOS: Length of hospital stay; NIS: National Inpatient Sample; OR: Odds ratio; UC: Ulcerative colitis.

regimen (use the vancomycin as a primary antibiotic, and a rapid decrease in steroid dosing)[11]. Using data from NIS, Ananthakrishnan *et al*[18] reported a significant increase in total colectomy odds from 1998 (OR 1.39, 95% CI: 0.81-2.37) to 2007 (OR = 2.51, 95%CI: 1.90-3.34) ($P = 0.03$) in IBD patients with CDI compared to IBD patients without CDI. Jen *et al*[17] using HES data for 2002/03 to 2007/08 found that IBD patients with CDI were exposed to a risk of undergoing gastrointestinal surgery or emergency colectomy 1.2 to 3 times higher than those with IBD alone.

In contradiction with previously mentioned studies, others communicated low rates of urgent colectomy in IBD patients with CDI. Thus, according to two recent studies from Cleveland Clinic's Digestive Disease Institute, United States, CDI in UC patients had no negative impact on colectomy risk within 3 mo of CDI diagnosis. In one study[20] including 78 patients (39 patients with UC and CDI, 39 with UC alone), 25 underwent colectomy, 12 of whom (48%) were among those with UC and CDI, and 13 (50.9%) with UC alone ($P = 0.81$). Also, in the second study[24], including 146 patients (45 with UC and CDI, 101 with UC without CDI), within 3 mo of index admission there was no significant difference concerning colectomy risk between UC patients with CDI and those without CDI; however, on long-term follow-up (one

Table 2 Long-term outcomes in inflammatory bowel disease patients with *Clostridium difficile* infection compared to those with inflammatory bowel disease alone

Ref.	Journal and year of publication	Study design and time frame	Outcome
Murthy *et al*[21] Canada	Aliment Pharmacol Ther 2012	In-patients; 2002-2008	UC patients with CDI was associated with increased adjusted 5-yr risk of mortality, but not of colectomy, as compared with UC without CDI
Navaneethan *et al*[24] United States	J Crohns Colitis 2012	Out-/in-patients; 2002-2007	One year following CDI: increased rates of ERV (37.8% *vs* 4%, $P = 0.001$) and colectomy (35.6% *vs* 9.9%, $P = 0.001$); escalation in medical therapy in 58.8% as compared to the prior year (12.9%) ($P = 0.0001$)
Jodorkovsky *et al*[19] United States	Dig Dis Sci 2010	In-patients; 2004-2005	One year following CDI: UC patients with CDI had increased rate of ERV (8 *vs* 1, $P = 0.012$), higher number of UC– related hospitalizations (58 *vs* 27, $P = 0.001$), and two-fold higher rates of colectomy (44.6% *vs* 25%, $P = 0.04$) compared to UC alone
Chiplunker *et al*[27] United States	Gastroenterology 2009	Case-control, in-patients; 2005-2006	One year following CDI: over half required an escalation in their IBD medical therapy, 46% had more hospitalisations, colectomy occured in 10.3% , and no mortality

CDI: *Clostridium difficile* infection; ERV: Emergency room visits; IBD: Inflammatory bowel disease; OR: Odds ratio; UC: Ulcerative colitis.

year), UC patients with CDI showed a significantly higher rate of colectomy than those without CDI. Kaneko *et al*[25] in a retrospective study from Yokohama City University Medical Center, Japan, reported that CDI did not have an impact on colectomy rate in their hospitalized UC patients with active disease, the difference in colectomy rate between UC+CDI patients (33.6%) and those without CDI (23.1%) being statistically insignificant (OR = 1.03, 95%CI: 0.41-2.63; $P = 0.94$). Two European studies[14,26] also reported low rates of colectomy (5% and 6%, respectively) in IBD patients co-infected with *C. difficile*. Recently, Murthy *et al*[21] found a similar rate of colectomy between UC patients with and without CDI (12% *vs* 9.8%, $P = 0.30$).

The different colectomy rates between single-center studies and those using nationwide data could be partially accounted for by differences in healthcare practice and threshold for surgery, response to CDI medical therapy, and data collection methods used[23].

Mortality rates are higher in IBD patients with CDI than in those without CDI or with CDI alone[13,16,17,21]. Among IBD patients, mortality is higher in UC than in CD[16]. All studies analysing nationwide databases reported high rates of mortality in IBD patients with CDI[13,16,17,21]. Nguyen *et al*[16] analyzed NIS discharge records from 1998 to 2004 and found that CDI was associated with a nearly four fold increase in mortality among hospitalized patients with UC (OR 3.79, 95% CI: 2.84-5.06) unlike those with CD (OR = 1.66, 95%CI: 0.75-3.66) as compared to non-IBD patients. Similarly, Ananthakrishnan *et al*[13], using also data from NIS, found that IBD patients with superimposed CDI had a four fold increase in mortality compared to patients hospitalized with IBD alone (OR = 4.7; 95%CI: 2.9-7.9) and twice higher than those with CDI alone (OR = 2.2, 95%CI: 1.4-3.4). In contrast to Nguyen *et al*[16] study, where mortality was higher only in UC, this study reported an increased mortality rate in both UC and CD patients. Jen *et al*[17] using HES data, reported that for the studied period (2002-2008), IBD patients with CDI were approximately six times more likely to die in hospital than those admitted for IBD alone (adjusted OR = 6.32, 95%CI: 5.67-7.04), and suggested that such high mortality rate may be partially due to increased number of all emergency gastrointestinal surgery and colectomy rates during admissions. Murthy *et al*[21] also reported a higher mortality rate in hospitalized UC patients co-infected with *C. difficile* than in uninfected UC patients (3.3% *vs* 0.38%, $P = 0.0001$).

Nevertheless, other studies showed a mortality rate for IBD patients with CDI similar to or not statistically higher than what was reported for non-infected IBD patients[14,18]. Thus, Bossuyt *et al*[14] registered no deaths among their patients with UC and CDI, while Ananthakrishnan *et al*[18] found a non-significant increase in the relative mortality risk in IBD patients with superimposed CDI from 1998 (OR = 2.38, 95%CI: 1.52-3.72) to 2007 (OR = 3.38, 95%CI: 2.66-4.29; $P = 0.15$).

LONG-TERM OUTCOMES

Few studies reported on long-term outcomes after an initial episode of CDI in IBD patients (Table 2)[19,21,24,27]. In a retrospective study including 47 patients with UC and CDI and 52 with UC without CDI, Jodorkovsky *et al*[19] reported that, over the year following the initial infection episode, a significant increase in the number of visits to the emergency room (8 *vs* 1, $P = 0.012$) was registered, as well as a higher number of UC-related hospitalizations (58 *vs* 27, $P = 0.001$) and a two-fold increase in colectomy rate (44% *vs* 25%, OR = 2.38, 95%CI: 1.01-5.6; $P = 0.04$) as compared to UC patients without CDI. Murthy *et al*[21], in a retrospective cohort study of UC patients with and without CDI, found that CDI was associated with higher adjusted 5-year risk of mortality [adjusted hazard ratio (aHR) = 2.40, 95%CI: 1.37-4.20], but not of colectomy (aHR = 1.18, 95%CI: 0.90-1.54). In another retrospective study[24], UC patients with CDI had significantly more UC-related emergency room visits (37 *vs* 4, $P < 0.001$) and a higher rate of colectomy (35.6% *vs* 9%, $P < 0.001$)

than those with UC alone in the year following initial infection. In addition, 55.8% of patients with UC and CDI had an escalation in medical therapy in the year after index infection admission as compared to 12.9% in the previous year ($P < 0.0001$). In a multivariate analysis for risk factors of colectomy, severe disease on endoscopy (OR = 16.7, 95%CI: 4.1-67.9; $P < 0.001$) and CDI (OR = 10.0, 95%CI: 2.7-36.3; $P < 0.001$) were found to be independently associated with colectomy within 1 year. Chiplunker *et al*[27] in a retrospective, case-control study on 81 patients with IBD comparing disease progression 1 year before and 1 year after the initial infection with *C. difficile*, found that 46% of patients had more hospitalizations and over a half of them (53%) required an escalation in medical therapy during the year following CDI. No deaths occurred during the 1 year follow-up.

Healthcare costs are higher in IBD patients with CDI than in those with IBD alone due to longer hospital stay, higher number of IBD-related hospitalizations, increased need for surgery, and hospital care charges[13,16]. Nguyen *et al*[16] found a mean cost increased by 46% and 63% for UC and CD patients, respectively, while Ananthakrishnan *et al*[13] reported higher increased hospital adjusted expenses of US$ 11406.

DISCUSSION

The majority of published studies (90%) found by searching Medline and other databases aim to assess CDI incidence in IBD patients, while only 10% of them report on outcome following infection. All studies reporting on outcome in IBD patients with concomitant CDI were retrospective, small single-center cohort studies or large nationwide database studies mostly conducted in North America and Europe. Apart from being retrospective, available studies on outcome have several other limitations such as incomplete information on disease severity, absence of reference to *C. difficile* diagnosis, antibiotic and immunomodulatory therapy. It should be underlined that most of analyzed studies relate to hospitalized IBD patients in the early 2000s, when enzyme immunoassay of stool for *C. difficile* toxins A and B has dominated the laboratory diagnosis of CDI, despite its low sensitivity.

Though few in number, studies reporting outcomes associated with CDI in IBD patients most often show that CDI has a negative impact both on short- and long-term IBD outcomes, increasing the need for surgery, morbidity and mortality rates, as well as healthcare costs[11,13,16-18,21,24,27]. Both major forms of IBD are under increasing risk for CDI, although patients with UC are most susceptible to infection and have more severe outcomes[15,16,19]. Short-term outcomes, defined as those measured within 30-90 d of index admission, include length of hospital stay, colectomy and mortality rates. Long-term outcomes, measured at least 1 year following index admission, include emergency room visits, UC-related hospitalizations, escalation in medical therapy, colectomy and mortality rates.

Studies report conflicting results on the length of hospital stay in IBD patients with concomitant CDI: some found similar stays[12,19], others shorter ones[14], while most of them reported longer hospitalization periods than in IBD patients without CDI or with CDI alone[11,13,16,17,21]. Similar or shorter hospitalization periods were reported by single-center studies[12,14,19], while longer stays were found in studies using nationwide databases[13,16,17,21]. Discrepancies between studies may be explained by disease severity and response to medical therapy in IBD patients included in the analyses.

Contrasting results have also been reported with regard to colectomy rates in IBD patients with CDI[11,13,14,17-21,24-26]. Thus, some studies analyzing nationwide data (US NIS HCUP, UK HES) reported high colectomy rates[13,18], while other single-center ones[20,24,25] found CDI to have no negative impact on colectomy rate in UC patients. There are discrepancies in what concerns colectomy rates even between studies using nationwide data in North America[13,18] and Europe[17], probably due to differences in data collection methods and threshold for surgery[23]. In addition, studies analyzing nationwide data for colectomy also include IBD patients admitted for elective surgery, a fact which can contribute to high colectomy rates[23]. Lower risks for colectomy, as reported by single-center North-American[20] and European[14,26] studies, may be partially explained by CDI prompt response to medical therapy, followed by clinical remission of IBD flare, thus preventing surgery[20].

Variable results regarding mortality rate in IBD patients with concomitant CDI are also to be noted[13,14,16-18,21]. High mortality rates reported by some studies using nationwide data[13,16,17] may be accounting for by to the increased use of colectomy in IBD patients with CDI[20], and also by the inclusion of all hospitalized patients who presumably have more severe disease than those from cohort studies[17,23].

Few studies reported long-term outcomes after an initial CDI episode in IBD (UC) patients. Two[19,24] reported an increased number of visits to the emergency room and in UC-related hospitalizations, and higher colectomy rate than in patients with UC alone in the year following initial infection. UC patients with CDI had an escalation in medical therapy 1 year after index admission as compared to the previous year[24,27]. Another study including UC hospitalized patients with and without CDI, found that those with associated CDI had a higher adjusted 5-year risk of mortality, but not of colectomy[21].

We may add that only one study aimed to identify predictive factors for severe outcomes (colectomy, death) associated with CDI in IBD patients[28]. Ananthakrishnan *et al*[28] in a retrospective study using multi-institutional electronic medical record database from two large referral hospitals over the period 1998-2010, reported a 4.4% colectomy and 15.2% mortality rates during 180-d follow-up of 294 IBD patients with CDI (mostly with UC), and found that among several demographic variables and laboratory parameters, only serum albumin below 3

g/dL, hemoglobin below 9 g/dL, and serum creatinine above 1.5 mg/dL were independent predictors of severe outcomes[28].

CDI IN IBD PATIENTS FOLLOWING SURGICAL INTERVENTION

Clostridium difficile enteritis usually occurs in IBD patients who have undergone colonic surgery, mainly proctocolectomy[29-31]. *C. difficile* enteritis is generally rare, although the number of cases reported in literature has recently increased[32]. The predisposition of IBD patients with previous colonic surgery to *C. difficile* enteritis may be accounted for by the colonization of the neo-terminal ileum with colonic-type bacterial flora[33], and phenotypic changes in the epithelium of pelvic ileoanal pouches[34]. *C. difficile* enteritis diagnosis and treatment are similar to that for colonic CDI. If some studies reported increased mortality among patients with *C. difficile* enteritis[35], other ones found low or even no mortality[29].

Clostridium difficile pouchitis has been reported in IBD patients with ileal pouch anal anastomosis (IPAA)[36-38]. Shen *et al*[36] found that 18.3% of their 115 patients with IPAA had CDI. Morphologic changes in the pouch epithelium secondary to prolonged exposures to fecal stream may favor CDI[34]. In addition, frequent antibiotic treatment for acute or chronic pouchitis is another risk factor for CDI in such patients[36]. Recently, Tyler *et al*[39] reported that genetic polymorphisms, particularly the NOD2insC risk allele, are associated with increased risk of developing pouch inflammation among patients with UC and IPAA. Treatment with vancomycin, tinidazole, or rifaximin has been used with benefit in many patients[37,38].

CONCLUSION

IBD patients with CDI are under a higher risk of worse outcomes than those without CDI. Because the available data are often conflicting and obtained from retrospective studies, further prospective multi-center studies are required to evaluate the impact of CDI on IBD outcomes. Until then, to improve patient outcome, clinicians should have a high index of suspicion for CDI in all IBD patients presenting with a disease flare in order to rapidly establish diagnosis and prompt treatment of infection.

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ORIGINAL ARTICLE

Impacts of common factors of life style on serum liver enzymes

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Abstract

AIM: To investigate the impacts of gender, age and factors of life style (alcohol, overweight, coffee and smoking) on serum liver enzymes.

METHODS: Serum alanine aminotransferase (ALT) and gamma-glutamyltransferase (GGT) were measured from 6269 apparently healthy individuals (2851 men, 3418 women, mean age 45 ± 12 years, range 25-74 years) in a national cross-sectional health survey. All subjects underwent detailed clinical examinations and interviews including the amount and pattern of alcohol use, coffee consumption and smoking habits.

RESULTS: In this population with a mean ± SD alcohol consumption of 65 ± 105 g/wk and body mass index (BMI) of 26.1 ± 4.3 kg/m^2, both ALT and GGT were significantly influenced by alcohol use ($P < 0.001$) and BMI ($P < 0.001$), whereas smoking increased only GGT ($P < 0.001$). A significant effect of age on ALT was seen in men ($P < 0.001$) whereas not in women. Significant two-factor interactions of alcohol use in men were observed with age (ALT: $P < 0.01$; GGT: $P < 0.001$) and BMI (GGT: $P < 0.05$). For ALT, a significant interaction also occurred between BMI and age ($P < 0.005$). In contrast, women showed significant interactions of alcohol use with BMI (GGT: $P < 0.05$), smoking (GGT: $P < 0.001$), and coffee consumption (GGT: $P < 0.001$).

CONCLUSION: Life-style associated changes in liver enzymes may reflect health risks, which should be considered in the definition of normal limits for liver enzymes.

Key words: Alcohol; Obesity; Aging; Smoking; Liver enzymes; Oxidative stress

Core tip: The present study among 6269 apparently healthy individuals shows that the early changes in serum alanine aminotransferase and gamma-glutamyltransferase levels show distinct age- and gender-dependent variation according to the amount of alcohol drinking and the presence or absence of overweight. Coffee consumption and smoking also modulate the enzyme levels with different sensitivities between genders. The data should be implicated in the assessment of health risks associated with such factors of life style and when revisiting the concept of normal limits in the clinical use of liver enzymes.

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INTRODUCTION

Since an increasing percentage of the general population consists of heavy drinkers[1,2], and over half suffer from overweight[3-7] adverse health effects resulting from such reasons constitute an inescapable problem in our society. Elevated serum liver enzymes and accumulation of fat in hepatic tissue are among the first manifestations in the sequence of events leading to morbidity and mortality due to alcohol drinking or excess body weight[8,9]. Consequently, there has been growing interest on the biological significance of the early-phase changes in serum liver enzyme activities and their clinical value as biomarkers of health and disease.

Recently, changes in serum alanine aminotransferase (ALT) and gamma-glutamyltransferase (GGT) have also attracted interest as prognostic parameters in a variety of extrahepatic conditions[10-15]. In both prospective and cross-sectional studies these enzymes have been observed to associate with diabetes, metabolic syndrome, and overall mortality[9,15-21]. Studies on the individual and joint impacts of various common factors of life style on ALT and GGT levels have, however, so far been limited. Similarly, the definitions of normal limits for these enzymes have remained as a matter of controversy and hampered the interpretation of the data in clinical trials.

In this work we measured serum ALT and GGT levels from a large age- and gender-stratified population of apparently healthy individuals with varying body mass index (BMI, kg/m^2) and with different levels of well-documented consumption of ethanol, coffee, and cigarette smoking. We also determined the normal limits for both enzymes based on the present sample of alcohol non-drinkers with normal body weight.

MATERIALS AND METHODS

Study protocol

The present data was collected from a cross-sectional population health survey (The National FINRISK studies) carried out in six different geographic areas in Finland. An age- and gender stratified random sample of 13437 individuals was first drawn from the population register according to an international WHO MONICA (Monitoring trends and determinants in cardiovascular disease) protocol. The survey included physical measurements, laboratory tests and detailed questionnaires on health status and alcohol intake, covering also information on current health behaviour, medical history and socioeconomic factors. The medical examinations were conducted using a previously described standardized protocol[22,23]. Measurements of height and weight were carried out and BMI was calculated as a measure of relative body weight. Serum ALT and GGT were measured by standard kinetic methods following recommendations of the European Committee for Clinical Laboratory Standards (ECCLS)[24] on an Abbott Architect clinical chemistry analyser (Abbott Laboratories, Abbott Park, IL, United States). Measurements of serum lipids [total cholesterol, high density lipoprotein (HDL) cholesterol, Low Density Lipoprotein (LDL) cholesterol, triglycerides] were carried out by enzymatic methods (Thermo Electron Corporation, Waltham, Massachusetts, United States). Serum C-reactive protein was measured using a sensitive immunoturbidimetric method (Orion Diagnostica, Espoo, Finland). All surveys were conducted in accordance with the Declaration of Helsinki according to the ethical rules of the National Public Health Institute. The approval for this study was received from the Coordinating Ethics Committee of the Helsinki Hospital District.

The present sample included data from the subjects who both filled out the questionnaire and attended the medical examination. The response rate was 65.5%. All participants were devoid of any apparent clinical signs of liver disease. In order to obtain a representative sample of apparently healthy individuals, exclusions were made for the following reasons: diagnosis of myocardial infarction (n = 236), stroke, cerebral haemorrhage or embolism (n = 193), diabetes or glucose-intolerance (n = 476), hypertension (n = 985), use of statins or lipid lowering agents (n = 364) or signs of active infection at the time of the study (n = 342). In addition, exclusions were made due to missing variables (n = 866). The final population thus comprised 6269 individuals: 2851 men and 3418 women (mean age 45 ± 12 years, range 25-74 years).

Alcohol consumption was assessed with detailed questions on the type of beverage consumed, the frequency of consumption, and the amount of ethanol-containing drinks consumed regularly during the past weeks and one year prior to the data collection. The amount of ethanol in different beverages was quantitated as follows: beer 12 g (1/3 L), strong beer 15.5 g (1/3 L), long drink 15.5 g (1/3 L), spirit 12 g (4 cL), wine 12 g (12 cL) and cider 12 g (1/3 L). A dose of 12 g of pure ethanol was considered as one standard drink. The persons who reported no current alcohol consumption were referred to as non-drinkers (n = 2048), moderate drinkers (n = 3993) consumed less than 280 g of ethanol (men) or less than 190 g of ethanol (women) per week, heavy drinkers (n = 228) consumed > 280 g per week (men) or > 190 g per week (women). Smoking and coffee consumption were assessed with a set of standardized questions. The data on smoking was expressed as the amount of cigarettes per day and coffee consumption was recorded as the number of cups of coffee per day.

Statistical analysis

Values are expressed as mean ± SD or mean ± 95%CI. Significance tests were carried out using ANOVA for multiple factors, Bonferroni *post hoc* test and appropri-

Table 1 Distribution of study participants (n = 6269) in subgroups according to age and gender n (%)

		Age group (yr)					
		25-29	30-39	40-49	50-59	60-69	70-74
Men							
n		325	714	727	671	345	69
Alcohol, g/wk							
	0	86 (26.5)	166 (23.2)	169 (23.2)	156 (23.2)	108 (31.3)	27 (39.1)
	< 280	222 (68.3)	510 (71.4)	515 (70.8)	466 (69.4)	219 (63.5)	39 (56.5)
	⩾ 280	17 (5.2)	38 (5.3)	43 (5.9)	49 (7.3)	18 (5.2)	3 (4.3)
BMI, kg/m^2							
	< 18.5	4 (1.2)	1 (0.1)	2 (0.3)	1 (0.1)	0 (0.0)	0 (0.0)
	⩾ 18.5 and < 25	171 (52.6)	260 (36.4)	236 (32.5)	208 (31.0)	101 (29.3)	20 (29.0)
	⩾ 25 and < 30	113 (34.8)	331 (46.4)	375 (51.6)	322 (48.0)	162 (47.0)	37 (53.6)
	⩾ 30	37 (11.4)	122 (17.1)	114 (15.7)	140 (20.9)	82 (23.8)	12 (17.4)
Smoking, cigarettes/d							
	0	189 (58.2)	446 (62.5)	476 (65.5)	469 (69.9)	274 (79.4)	65 (94.2)
	1-10	68 (20.9)	94 (13.2)	67 (9.2)	49 (7.3)	22 (6.4)	3 (4.3)
	⩾ 11	68 (20.9)	174 (24.4)	184 (25.3)	153 (22.8)	49 (14.2)	1 (1.4)
Coffee, cups/d							
	0	62 (19.1)	81 (11.3)	55 (7.6)	59 (8.8)	24 (7.0)	5 (7.2)
	1-4	143 (44.0)	248 (34.7)	239 (32.9)	246 (36.7)	180 (52.2)	39 (56.5)
	⩾ 5	120 (36.9)	385 (53.9)	433 (59.6)	366 (54.5)	141 (40.9)	25 (36.2)
Women							
n		461	861	860	795	365	76
Alcohol, g/wk							
	0	190 (41.2)	356 (41.3)	308 (35.8)	289 (36.4)	155 (42.5)	38 (50.0)
	< 190	267 (57.9)	492 (57.1)	533 (62.0)	489 (61.5)	203 (55.6)	38 (50.0)
	⩾ 190	4 (0.9)	13 (1.5)	19 (2.2)	17 (2.1)	7 (1.9)	0 (0.0)
BMI, kg/m^2							
	< 18.5	23 (5.0)	12 (1.4)	7 (0.8)	1 (0.1)	0 (0.0)	1 (1.3)
	⩾ 18.5 and < 25	305 (66.2)	508 (59.0)	488 (56.7)	311 (39.1)	126 (34.5)	25 (32.9)
	⩾ 25 and < 30	92 (20.0)	245 (28.5)	245 (28.5)	325 (40.9)	153 (41.9)	31 (40.8)
	⩾ 30	41 (8.9)	96 (11.1)	120 (14.0)	158 (19.9)	86 (23.6)	19 (25.0)
Smoking, cigarettes/d							
	0	314 (68.1)	616 (71.5)	616 (71.6)	652 (82.0)	326 (89.3)	71 (93.4)
	1-10	109 (23.6)	157 (18.2)	141 (16.4)	75 (9.4)	21 (5.8)	2 (2.6)
	⩾ 11	38 (8.2)	88 (10.2)	103 (12.0)	68 (8.6)	18 (4.9)	3 (3.9)
Coffee, cups/d							
	0	145 (31.5)	152 (17.7)	71 (8.3)	62 (7.8)	22 (6.0)	5 (6.6)
	1-4	235 (51.0)	440 (51.1)	409 (47.6)	445 (56.0)	240 (65.8)	59 (77.6)
	⩾ 5	81 (17.6)	269 (31.2)	380 (44.2)	288 (36.2)	103 (28.2)	12 (15.8)

ate covariates as indicated. Logarithmic transformations of ALT and GGT data were used to obtain non-skewed distributions with homogeneity of variance. Correlations were calculated using Pearson product-moment correlation coefficients. The differences in partial correlations were analyzed with the *Z*-test for correlation coefficients. Calculations of reference limits based on the population of normal-weight non-drinkers were carried out according to previously described nonparametric methods and Dixon's test for detection and exclusion of outliers[25,26]. Age of 40 years was used as a cut-off for group stratification based on previous findings showing an increase in the 97.5 percentile of GGT in both genders at the age of about 40 years[26]. The analyses were carried out with the use of Analyse-it v 2.21 for Microsoft Excel (Leeds, United Kingdom) and SPSS 21.0 (SPSS Inc., Chicago, IL) for Windows statistical software. A P value < 0.05 was considered statistically significant.

RESULTS

This study cohort of apparently healthy individuals consisted of 2851 men and 3418 women, who participated in a national cross-sectional health survey. The data on alcohol consumption indicated that 32.7% of the population were non-drinkers, 63.7% were moderate drinkers and 3.6% were heavy drinkers. The mean ± SD alcohol consumption was 65 ± 105 g/wk: men 99 ± 137 g/wk, women 37 ± 53 g/wk. In this material with a mean BMI of 26.1 ± 4.3 kg/m^2 (men 26.7 ± 3.9 kg/m^2; women 25.6 ± 4.5 kg/m^2), 16.4% of the subjects were obese (BMI > 30 kg/m^2) and 38.8% showed BMI levels between 25 and 30 kg/m^2 indicating overweight. Smokers comprised 28.0% of the population. The demographic characteristics of the study participants, as further classified according to age and gender, are summarized in Table 1.

The lower and upper normal limits for ALT and

Table 2 Lower and upper normal limits for alanine aminotransferase and gamma-glutamyltransferase based on non-drinkers with normal weight

	Normal weight non-drinkers			Reference[1]	
	n	Lower limit U/L	Upper limit U/L	Lower limit U/L	Upper limit U/L
ALT					
Men	162	10	47	10	70
Women	523	8	37	10	45
GGT					
Men	162	11	52		
< 40 yr	55	10	48	10	80
⩾ 40 yr	107	12	53	15	115
Women	522	8	42		
< 40 yr	228	8	34	10	45
⩾ 40 yr	294	9	47	10	75

[1]Commonly used values in Nordic countries based on NORIP data[26].

GGT as defined here by calculating 2.5th and 97.5th percentiles of the data based on normal weight non-drinkers are shown in Table 2. The observed upper normal limits for both enzymes were found to be significantly lower than the currently used limits in Nordic countries used as reference in the present comparisons[26].

In the total study material, significant effects of alcohol consumption ($P < 0.001$), BMI ($P < 0.001$), and age ($P < 0.001$) were noted for both ALT and GGT. In the analyses for main effects and interactions between the study variables, the levels of ALT and GGT in both genders were found to be significantly influenced by alcohol use (ALT: $P < 0.05$ for men; $P < 0.001$ for all other comparisons) and BMI ($P < 0.001$) (Table 3). There was also a significant main effect of age on GGT ($P < 0.001$) and on ALT in men ($P < 0.001$), whereas not in women (Figure 1, Table 3). Highest ALT levels in men occurred in those aged 25-50 years whereas in women such age-dependent variation was not observed (Figure 1). The highest GGT values were observed in age groups 50-60 years (men) and 60-70 years (women) (Figure 1). Smoking significantly influenced GGT levels in both genders ($P < 0.001$) (Table 3).

The impact of ethanol intake in men was found to become significantly more pronounced upon increasing age (Figure 2, Table 3). In men over 40 years alcohol consumption exceeding 16 drinks per week was a stronger determinant of GGT activities than in those below 40 (Figure 2). In women, such interaction was not observed.

The effects of increasing body weight on ALT and GGT levels in the different age-categories are shown in Figure 3. Interestingly, in men below 40 years, overweight was found to be a stronger inducer of ALT activities than in those above 40 years. In contrast such interaction between BMI and age was not found among women (Table 3). The analysis of the effect of alcohol drinking in the different BMI-based subgroups showed that the presence of overweight or obesity significantly potentiates GGT activities both in men and women who consume alcohol (Figure 4). Analyses of two-factor interactions between

Table 3 Summary of main effects and two-factor interaction statistics for alanine aminotransferase and gamma-glutamyltransferase

	Men		Women	
	ALT	GGT	ALT	GGT
Main effects				
Ethanol	< 0.050	< 0.001	< 0.001	< 0.001
BMI	< 0.001	< 0.001	< 0.001	< 0.001
Age	< 0.001	< 0.001	NS	< 0.001
Smoking	NS	< 0.001	NS	< 0.001
Coffee	NS	NS	NS	< 0.050
Two-factor interaction				
Ethanol × age	0.008	0.001	0.295	0.197
Ethanol × BMI	0.301	0.038	0.537	0.048
Ethanol × smoking	0.398	0.094	0.794	0.001
Ethanol × coffee	0.534	0.260	0.138	0.001
BMI × age	0.003	0.067	0.502	0.788
BMI × smoking	0.878	0.342	0.904	0.690
BMI × coffee	0.430	0.653	0.261	0.090
Age × smoking	0.831	0.089	0.558	0.342
Age × coffee	0.095	0.117	0.018	0.061

The analyses of main effects and two-factor interactions were carried out using ANOVA on SPSS 21.0 for Windows statistical software. Alcohol use, BMI, smoking and coffee consumption were used as covariates, as appropriate. NS: Not significant; LT: Alanine aminotransferase; GGT: Gamma-glutamyltransferase; BMI: Body mass index.

the study variables also showed statistically significant interactions of alcohol use and smoking (GGT: $P < 0.001$) as well as between alcohol use and coffee consumption especially among women (GGT: $P < 0.001$).

The correlations between ALT, GGT and various metabolic and inflammatory markers are summarized in Table 4. Significant correlations emerged between the liver enzymes and the lipid profile, C-reactive protein and indices of overweight. However, there were also distinct differences in the correlation coefficients observed between the liver enzymes and the other biomarkers when comparing men and women or subjects below or above 40 years of age. Stronger correlations between liver enzymes and indices of lipid status (cholesterol, triglycerides) were found in men. Significant correlations also occurred between the liver enzyme levels and waist circumference, which especially in case of GGT among men over 40 years was slightly stronger than the corresponding correlation with BMI (Table 4).

DISCUSSION

Our data among a large cross-sectional sample of apparently healthy individuals shows age- and gender-dependent interactions between alcohol use, BMI, smoking and serum liver enzymes, which have recently been suggested as important disease risk markers in both hepatic and extrahepatic conditions[12,27]. The data also suggests distinct differences in the reactivities of ALT and GGT towards the metabolic burdens created by the various factors of life style.

In current societies, alcohol and obesity-related health

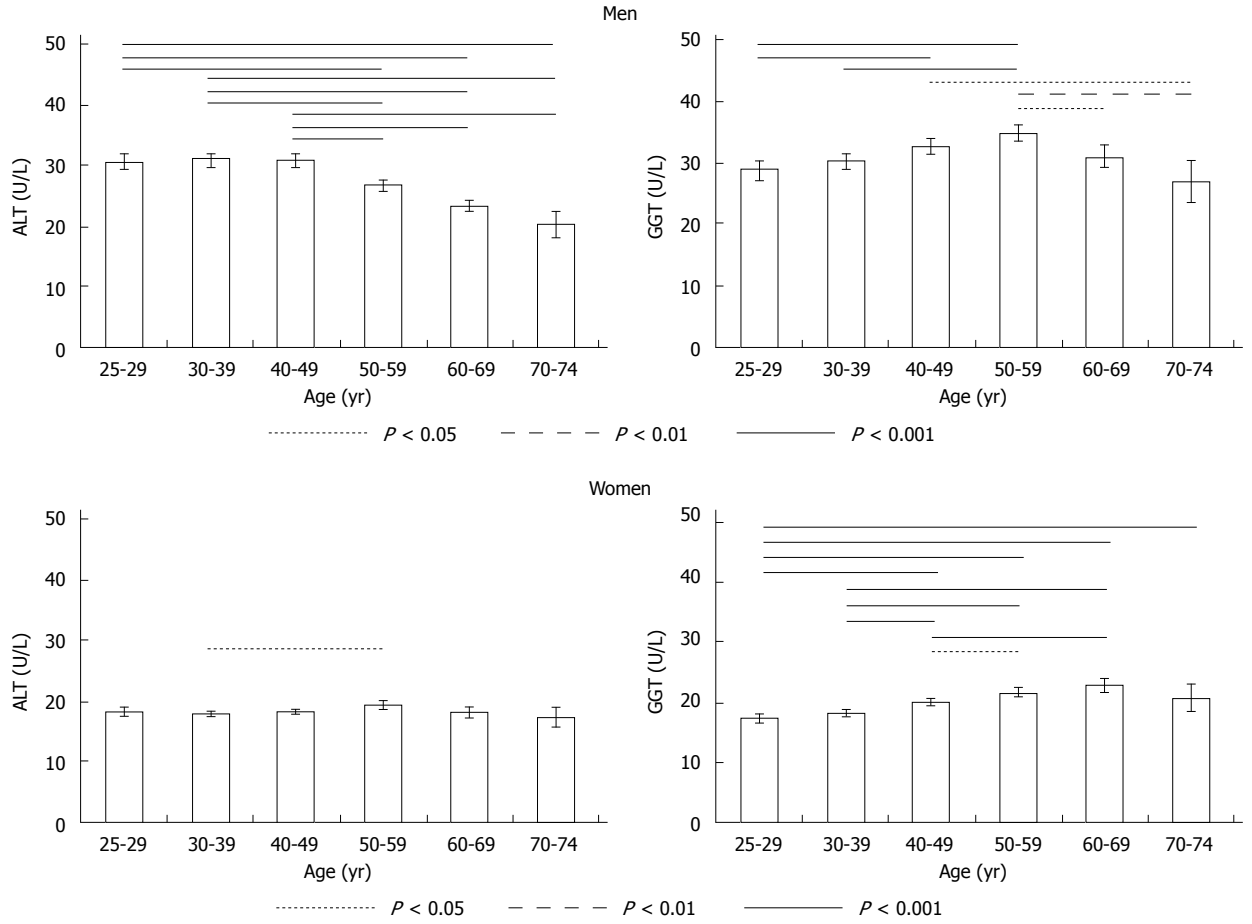

Figure 1 Alanine aminotransferase and gamma-glutamyltransferase levels (geometric mean ± 95%CI) in men and women classified to subgroups according to age. Horizontal lines indicate significant differences between groups, as assessed by ANOVA with Bonferroni *post hoc* test. Alcohol intake (drinks/wk), BMI (kg/m^2), smoking (cigarettes/d), and coffee consumption (cups/d) were used as covariates. ALT: Alanine aminotransferase; GGT: Gamma-glutamyltransferase.

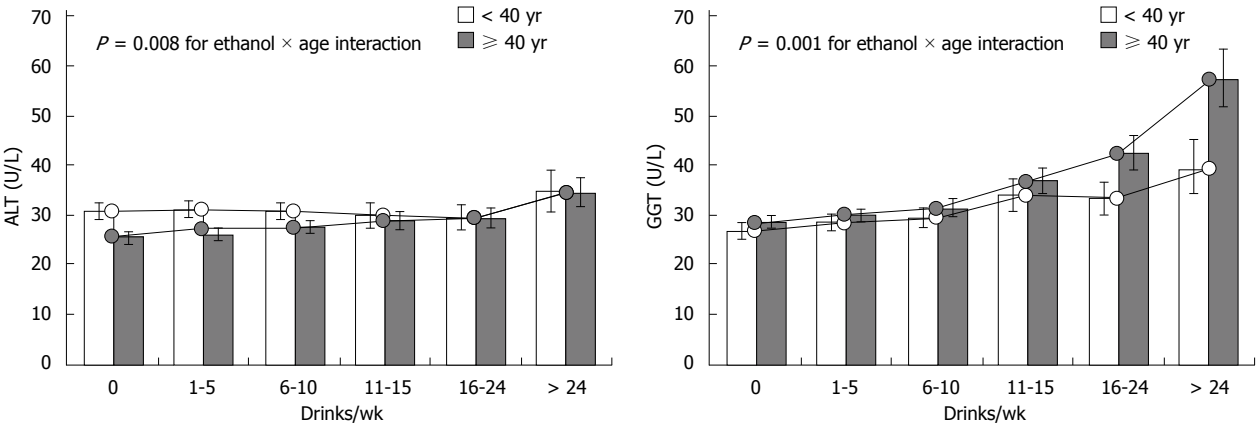

Figure 2 Interactions of ethanol intake with age on alanine aminotransferase and gamma-glutamyltransferase levels in men. An aggravated effect of ethanol was seen in those who were over 40 years of age and consumed over 16 drinks of alcohol per week. In women, the interaction between alcohol use and age was not significant (Table 3). Smoking and coffee consumption were used as covariates. ALT: Alanine aminotransferase; GGT: Gamma-glutamyltransferase.

problems are often co-existing and may function in a synergistic manner[9,16,21,28]. GGT has previously been shown to readily increase among alcohol consumers with obesity[16] as well as in heavily smoking alcohol users[17], which may be related with the pivotal role of GGT in the metabolism of glutathione (GSH)[29-34]. Mild GGT elevations may be considered a sign of a need to maintain intracellular GSH levels under conditions of oxidative stress[14,29,30,32-34]. In turn, alterations in ALT activities likely reflect disturbed liver cell integrity[27].

According to the present data the upper normal limits of ALT and GGT enzymes in their clinical use as disease

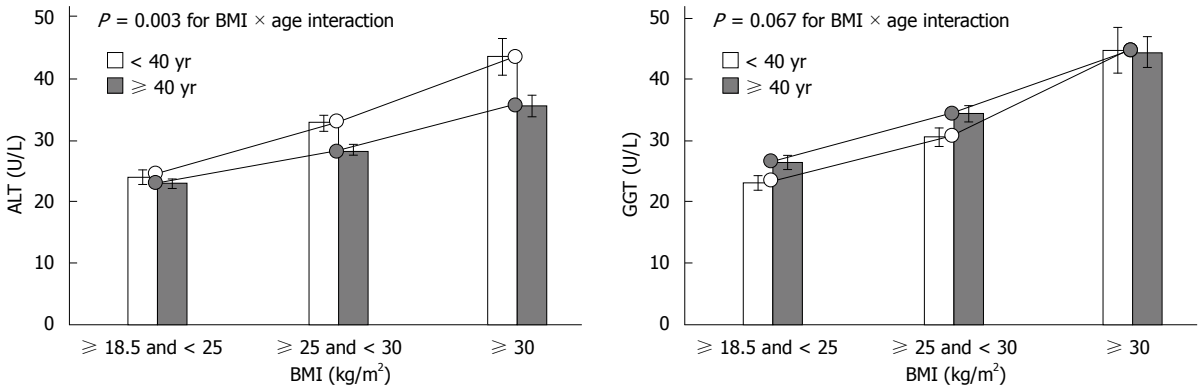

Figure 3 Interactions of body mass index with age on alanine aminotransferase and gamma-glutamyltransferase levels. A significant interaction was noted only on alanine aminotransferase (ALT) levels in men below 40 years of age. Alcohol intake, smoking and coffee consumption were used as covariates. BMI: Body mass index.

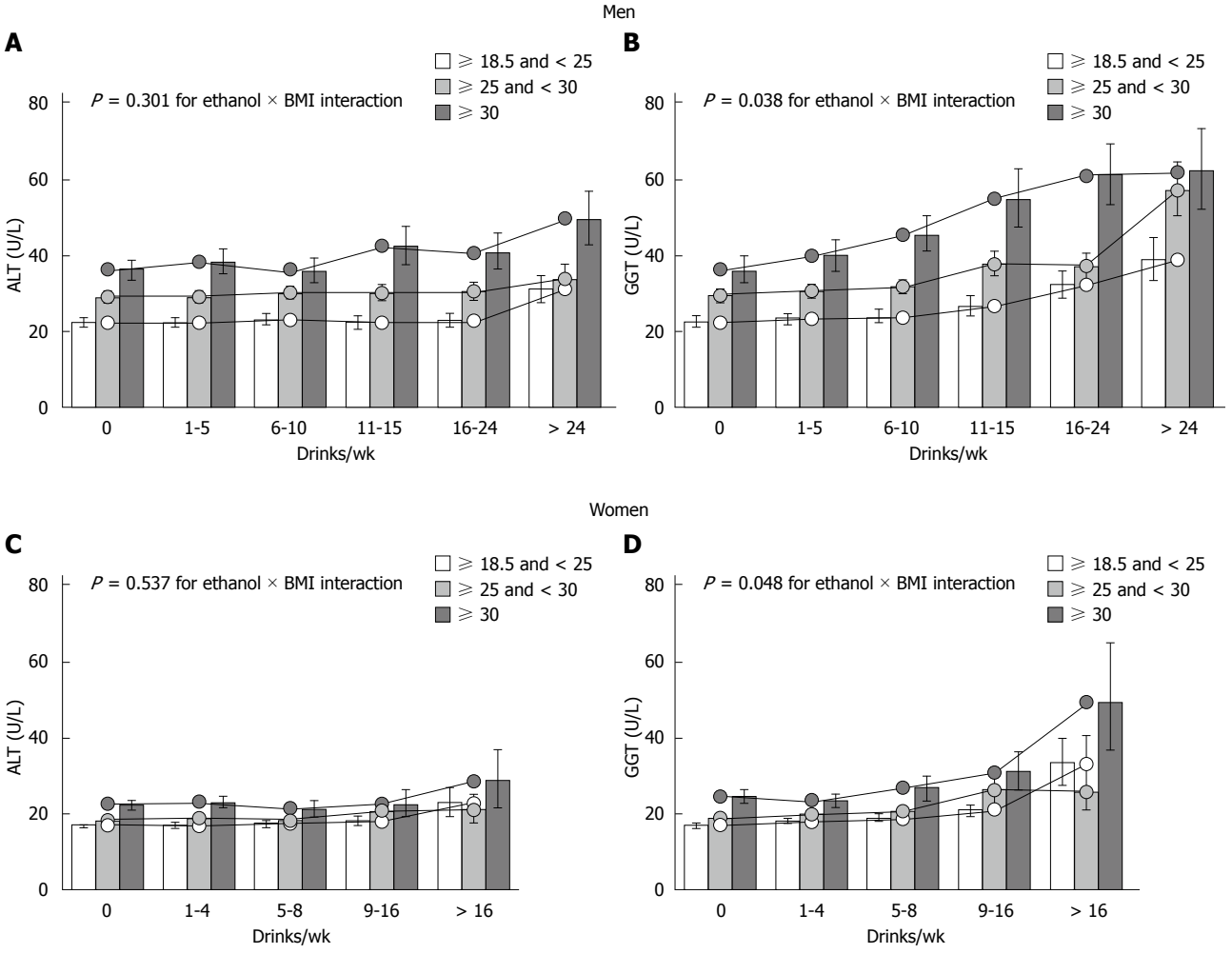

Figure 4 Interactions of ethanol intake with body mass index on alanine aminotransferase and gamma-glutamyltransferase levels. Significant interactions in both men and women were noted for gamma-glutamyltransferase (GGT), whereas not for alanine aminotransferase (ALT). Covariates used were age (years), smoking, and coffee consumption. BMI: Body mass index.

biomarkers should be markedly lower than those currently used in most countries[35,36]. Despite of the fact that biomarker reference intervals are crucial tools to differentiate between healthy and diseased subjects, as yet we share no widely accepted upper normal limits even for these most common liver enzymes. This may have been due to lack of knowledge on dose responses between ethanol intake and biomarker levels, inconsistencies regarding the definition of safe levels of ethanol intake and ignorance of excess body weight in different sample populations. For successful implementation of early treatment programs to reduce alcohol- and obesity-induced morbidity, correct definitions of reference intervals and greater harmonization of analytical goals for biomarkers

Table 4 Correlations between study variables

	Men (n = 2851)		Women (n = 3418)	
	ALT	GGT	ALT	GGT
ALT		0.543[a]		0.483[a]
Total cholesterol	0.182[1,a]	0.347[1,a]	0.105[1,a]	0.193[1,a]
HDL	-0.163[a]	-0.017	-0.098[a]	-0.045[b]
LDL	0.151[a]	0.252[1,a]	0.118[a]	0.182[1,a]
Triglycerides	0.266[1,a]	0.327[1,a]	0.178[1,a]	0.229[1,a]
CRP	0.142[a]	0.280[a]	0.104[a]	0.238[a]
BMI	0.378[1,a]	0.351[1,a]	0.256[1,a]	0.284[1,a]
Waist circumference	0.367[1,a]	0.395[1,a]	0.253[1,a]	0.303[1,a]
	Men < 40 yr (n = 1039)		Men ⩾ 40 yr (n = 1812)	
	ALT	GGT	ALT	GGT
ALT		0.592[a]		0.538[a]
Total cholesterol	0.297[1,a]	0.391[1,a]	0.164[1,a]	0.298[1,a]
HDL	-0.201[a]	-0.083[b]	-0.139[a]	0.003
LDL	0.282[1,a]	0.332[1,a]	0.110[1,a]	0.178[1,a]
Triglycerides	0.322[a]	0.373[1,a]	0.256[a]	0.301[1,a]
CRP	0.180[a]	0.267[a]	0.137[a]	0.273[a]
BMI	0.451[1,a]	0.446[1,a]	0.351[1,a]	0.284[1,a]
Waist circumference	0.459[1,a]	0.463[1,a]	0.352[1,a]	0.336[1,a]
	Women < 40 yr (n = 1322)		Women ⩾ 40 yr (n = 2096)	
	ALT	GGT	ALT	GGT
ALT		0.450[d]		0.495[d]
Total cholesterol	0.050	0.087[b]	0.099[d]	0.154[d]
HDL	-0.114[d]	-0.054[a]	-0.096[d]	-0.057[b]
LDL	0.082[b]	0.104[d]	0.106[d]	0.139[d]
Triglycerides	0.083[1,b]	0.115[1,d]	0.217[1,d]	0.249[1,d]
CRP	0.044[1]	0.154[1,d]	0.148[1,d]	0.302[1,d]
BMI	0.267[d]	0.291[d]	0.231[d]	0.236[d]
Waist circumference	0.242[d]	0.292[d]	0.244[d]	0.267[d]

[a]P < 0.05 *vs* Control, [b]P < 0.01 *vs* Control, [d]P < 0.001 *vs* Control. [1]Statistically significant differences (P < 0.05) in correlation coefficients between men and women or those below or above 40 years of age (Z-test). ALT: Alanine aminotransferase; GGT: Gamma-glutamyltransferase; HDL: High density lipoprotein; LDL: Low density lipoprotein; CRP: C-Reactive protein; BMI: Body mass index.

with predictive value should be of utmost importance.

In the present study population, 40 years of age was used as a cut-off for group stratification based on previous findings, which have shown an increase in GGT levels in both genders at this age point[26]. Among men over 40 years, alcohol consumption even in amounts which were below the current limits of heavy drinking appeared to pose a risk towards elevated ALT and GGT levels, suggesting an increased susceptibility to ethanol-induced adverse health effects upon increasing age[31,37]. Intriguingly, a recent large United States population-based study also suggested relatively high mortality rates among older men consuming ethanol[38]. Although women in general are known to be more susceptible to ethanol-induced morbidity, the concept of safe limits for ethanol intake in different age categories of men also appears to need further attention.

The present findings also underscore the impact of excess body weight in the biological interactions influencing liver enzyme status. Young men presenting with overweight or obesity were found to show rather high baseline ALT levels even in the absence of any other apparent risk factors. The correlations between the lipid status and liver enzymes were also strong among young men, possibly indicating accumulation of adverse health effects due to a lifestyle involving overconsumption of the Western diet[39-41]. Previously, a high risk of liver injury in combination of obesity and alcohol use has been observed in studies among older age groups[42]. In experimental animals aging also promotes the development of diet-induced steatohepatitis and induction of serum amino transferase levels[39]. It is likely that alcohol intake by subjects with overweight stimulates oxidative stress in an additive and more striking manner, as also supported here by the findings in GGT activities[43,44]. Alcohol has a high energy content and in experimental animals the adverse effects of ethanol are aggravated by high-fat-diets[45]. Moreover, genotypic differences in alcohol-metabolizing enzymes could also contribute to the risk of gaining body weight in some alcohol consumers[46].

In accordance with previous data[10,17] the present observations also point to a significant synergistic effect of smoking and alcohol use in increasing GGT levels. However, while previous work reported such effects in male construction workers[10] our data suggests even stronger interactions among women. This could, however, be explained by the relatively lower quantities of smoking in the present material. In addition, coffee consumption was found to interact with GGT levels such that a high intake of coffee (⩾ 5 cups/d) in those with the most abundant amounts of ethanol intake was more likely to be associated with atypically low GGT levels indicating a possible protective effect of coffee towards alcohol-induced liver damage and oxidative stress[47-49].

Increased ALT and GGT levels commonly co-occur with accumulation of triglycerides and liver steatosis and compelling evidence from the past decade have also linked such phenomena with extrahepatic health risks, such as type 2 diabetes, metabolic syndrome, insulin resistance and cardiovascular morbidity[12,15,21,50-54]. Serum liver enzyme activities may even predict mortality from cardiovascular or cerebrovascular events[11,15,41,51]. While the specific mechanisms underlying such observations have remained unclear, it should be noted that recent studies have indicated a role for GGT as a link between fatty liver and development of early atherosclerosis due to the ability of GGT to trigger iron-dependent oxidation of LDL also in coronary plaques[51]. Therefore, it is notable that the present data also indicates a strong correlation between LDL cholesterol and GGT levels, especially in men.

The cross sectional setting of the survey can be kept as a limitation of this study as some of the biomarkers used may have day to day variation. Lack of follow-up data also prevents analyses on the specific relationships between enzyme elevations and duration of drinking or obesity status, which clearly warrant future prospective studies. Also disease status was self-reported and thus the exclusions might not be fully accurate causing conserva-

tive estimate of healthy population. It is also possible that the alcohol recall techniques overestimate the proportion of those not drinking alcohol at all[55]. However, all these issues most likely have diluting effects to the observed results and the real associations and interactions might be even stronger. It should also be noted that due to both financial and ethical considerations, analyses of hepatitis serology were not carried out in the present material. However, due to the low prevalence of viral hepatitis in Finland (observed rates of 1-2 cases/10000 blood donors per year) this should not create a significant confounding factor here.

Taken together, the present data provides novel information on the individual contributions of various factors of life style on the early-phase activation of liver enzymes and shows that even moderate drinking may lead to significant enzyme elevations in an age-, gender-, and BMI-dependent manner. Current data should be considered in the definition of more accurate safe limits of ethanol intake in different demographic categories and in the definition of normal values for liver enzymes. The possible mechanistic roles of liver enzymes as pathophysiological links between hepatic and extrahepatic disease manifestations warrant further studies.

COMMENTS

Background

The global burden of liver diseases due to excessive alcohol intake and obesity has shown a dramatic increase during the past decades. Measurements of liver enzymes, gamma-glutamyltransferase (GGT) and alanine aminotransferase (ALT), are widely used tools for detecting liver problems. However, the interpretation of the enzyme data in clinical work has been problematic due to the lack of knowledge on their early-phase responses towards the various factors of life style and the cut-offs defining normality in the assays.

Research frontiers

Serum ALT and GGT activities are known to increase as a result of alcohol use and increasing body weight. In this study among 6269 healthy volunteers, the authors demonstrate distinct age- and gender-dependent effects of alcohol use, overweight, coffee consumption and smoking on the activities of these enzymes.

Innovations and breakthroughs

The present studies demonstrate both individual and joint effects of the various factors of life style in creating increased activities of serum liver enzymes. The data also describes the lower and upper normal limits for ALT and GGT based on the present population of normal-weight non-drinkers.

Applications

By further understanding of the influences created by the various factors of life style and by more detailed definitions of liver enzyme normal limits, the clinical value of serum liver enzyme determinations can be markedly improved.

Terminology

Serum ALT and GGT are both commonly used in the diagnosis of liver diseases and have recently received increasing attention also as biomarkers of prognostic significance in extrahepatic conditions.

Peer review

This study investigated the relationships of liver enzymes and anthropometric and lifestyle factors in a general population of apparently healthy individuals. The results are interesting and may provide new insights into the clinical use of serum GGT and ALT as biomarkers of liver status.

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ORIGINAL ARTICLE

Osthol attenuates hepatic steatosis *via* decreased triglyceride synthesis not by insulin resistance

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Abstract

AIM: To evaluate the effects of osthol on intrahepatic fat synthesis, β-oxidation, inflammation, and insulin resistance by multifaceted analysis.

METHODS: Sprague-Dawley rats (n = 30) were randomly divided into control, non-alcoholic fatty liver disease (NAFLD), and osthol groups. NAFLD and osthol groups were fed with a high-fat diet for 14 wk. After 8 wk of the high-fat diet, the osthol group also received osthol 20 mg/kg orally 5 times/wk. To assess the insulin resistance, oral glucose tolerance was performed at the end of 14 wk. Immunohistochemical (4-HNE, F4/80) and hematoxylin and eosin (HE) staining were performed on liver tissue extracts after animal sacrifice at 14 wk. SREBP1c, FAS, SCD-1, PPAR-α, CROT, MCP-1, IRS-1, and IRS-2 mRNA expressions were assessed with reverse transcription-polymerase chain reaction.

RESULTS: HE staining revealed that, compared with the NAFLD group, the osthol group showed significantly decreased intrahepatic fat content (39.4% *vs* 21.0%; P = 0.021). SREBP1c expression in the NAFLD group increased compared to controls (P = 0.0001), while osthol treatment decreased SREBP1c expression compared with the NAFLD group (P = 0.0059). In the osthol group, intrahepatic FAS and SCD-1, which act downstream of SREBP1c, decreased significantly compared with the NAFLD group. Moreover, PPAR-α expression in the osthol group was also significantly higher than in the NAFLD group (P = 0.0147).

CONCLUSION: Osthol treatment attenuated liver steatosis by decreasing *de novo* liver triglyceride synthesis and had nominal effects on insulin resistance and liver inflammation.

Key words: Osthol; Non-alcoholic fatty liver disease; Sterol regulatory element binding protein

Core tip: Nonalcoholic fatty liver disease is considered as a consequence of "multi-hit" processes. Osthol, a coumarin compound, has anti-inflammatory effects on various diseases. However, there is no multi-faceted and comprehensive evaluation of its effects. The current study evaluated effects of osthol on intrahepatic fat synthesis, β-oxidation, inflammation, and insulin resistance by multifaceted analysis.

Nam HH, Jun DW, Jeon HJ, Lee JS, Saeed WK, Kim EK. Osthol attenuates hepatic steatosis *via* decreased triglyceride

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) has a worldwide prevalence of about 20%-30% and is among the most common causes of chronic liver disease[1,2]. Approximately 50%-60% of non-alcoholic steatohepatitis (NASH) patients have accompanying complications such as diabetes mellitus, cardiovascular disease, and hyperlipidemia[1]. NAFLD and NASH are also strongly associated with insulin resistance and obesity[3]. In hepatocytes, increased fatty acid oxidation increases oxidative stress and leads to apoptosis, which is also thought to be involved in NASH pathophysiology. However, until recently, it is thought that a cell's response to oxidative stress is more critical in determining its fate than the amount of oxidative stress. Furthermore, NAFLD is also regarded as a "multi-hit" disease[4,5] and could be the consequence of multiple highly entwined mechanisms such as insulin resistance, oxidative stress, mitochondrial insufficiency, endoplasmic reticulum stress, and apoptosis. Understanding these intricate mechanisms leading to NAFLD progression could provide a useful insight to understand NAFLD.

Insulin resistance together with oxidative stress has an important role in NAFLD pathophysiology[6-8]. An improvement in insulin resistance could decrease the incidence of NAFLD and NASH; however, hepatic insulin sensitizers have not provided significantly beneficial results in clinical trials[9-11]. Moreover, although antioxidant treatment improved NAFLD histology in clinical trials[6], the long-term effects of antioxidant treatments need further evaluation[12]. *Cnidium monnieri* fruits are used in traditional Chinese medicine. Osthol, the active constituent of *Cnidium monnieri* extracts, has anti-inflammatory and hepatic fat oxidizing properties. For instance, in a rat model of fatty liver, osthol not only decreased the fasting blood glucose and hepatic fat content, but also improved insulin resistance[13]. Zhang *et al*[14] also reported that osthol treatment decreased hepatic fat content by increasing the expression of hepatic peroxisome proliferator-activated receptor (PPAR)-α/γ. In the alcoholic fatty liver model, osthol treatment led to increased superoxide dismutase (SOD) activation and decreased oxidative stress[15]. These results suggest that osthol treatment not only reduces hepatic fat content and oxidative stress but also improves insulin resistance; however, the histological improvement in inflammation and fibrosis still requires further evaluation. Although the fat oxidizing effects of osthol have already been well studied, none of the previous studies evaluated the effects of osthol on liver inflammation and fibrosis simultaneously with the fat oxidizing effects. As multiple cellular mechanisms, such as hepatic fat synthesis, oxidative stress, inflammation, insulin resistance, and cellular adaptation, could all be involved in NAFLD pathophysiology, a comprehensive evaluation of osthol efficacy in NAFLD pathophysiology is needed. Therefore, the aim of the current study was to evaluate the precise mechanism and effects of osthol treatment on these multiple mechanism simultaneously.

MATERIALS AND METHODS

Experimental design

A total of 30 Sprague-Dawley (SD) rats (4-wk-old) were purchased from Orient Animal Laboratory, Seoul, South Korea and were randomly divided into 3 groups: control, NAFLD and osthol. The control group was fed normal chow while a combination of 60% high-fat (HF) diet and 20% fructose was provided to NAFLD and osthol groups. The fructose was provided in drinking water to NAFLD and osthol groups. From the 9th to 14th wk the NAFLD and osthol groups were treated orally 5 times/wk with normal saline (200 μL) and osthol 20 mg/kg, respectively[14,16,17] (dissolved in sodium carboxymethyl cellulose and later in normal saline to make 200 μL volume). After 14 wk, anesthetized animals were euthanized by thoracotomy and blood samples were withdrawn by cardiac puncture. The liver tissues were extracted for polymerase chain reaction (PCR), hematoxylin and eosin (HE) staining and immunohistostaining analysis. The experimental protocol was approved by Hanyang Institutional Animal Care and Use Committee (HY-IACUC-11-064).

Body weight assessment

The body weight of the animals was measured weekly from the start of the experiment to just before sacrifice to evaluate the changes in body weight.

Oral glucose tolerance test and serum aspartate transaminase/alanine transaminase

An oral glucose tolerance test (OGTT) was performed as follows[18]: briefly, after overnight fasting, the blood glucose level was measured through the tail vein at 0, 30, 60, 90, and 120 min after an oral glucose load of 2 g/kg body weight. After 14 wk, serum aspartate transaminase (AST) and alanine transaminase (ALT) were measured from the blood of sacrificed animals using a biochemical analytical system (Hitachi-747; Hitachi, Tokyo, Japan).

Histology and immunohistochemistry

Formalin-fixed paraffin embedded sections of liver tissue samples were stained with HE for microscopic analysis. To assess hepatic steatosis, the tissue sections were scored for activity (degree of inflammation) and stage (degree of fibrosis) of disease according to the histological grading and staging systems, respectively. Hepatic steatosis was graded as follows: < 5% (score, 0); 5%-33%

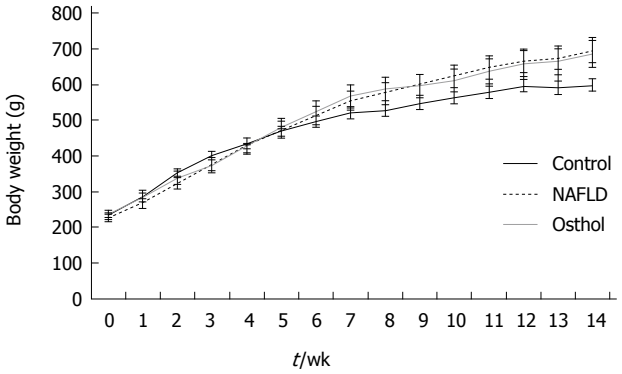

Figure 1 Changes in body weight of control, non-alcoholic fatty liver disease and osthol groups. NAFLD: Non-alcoholic fatty liver disease.

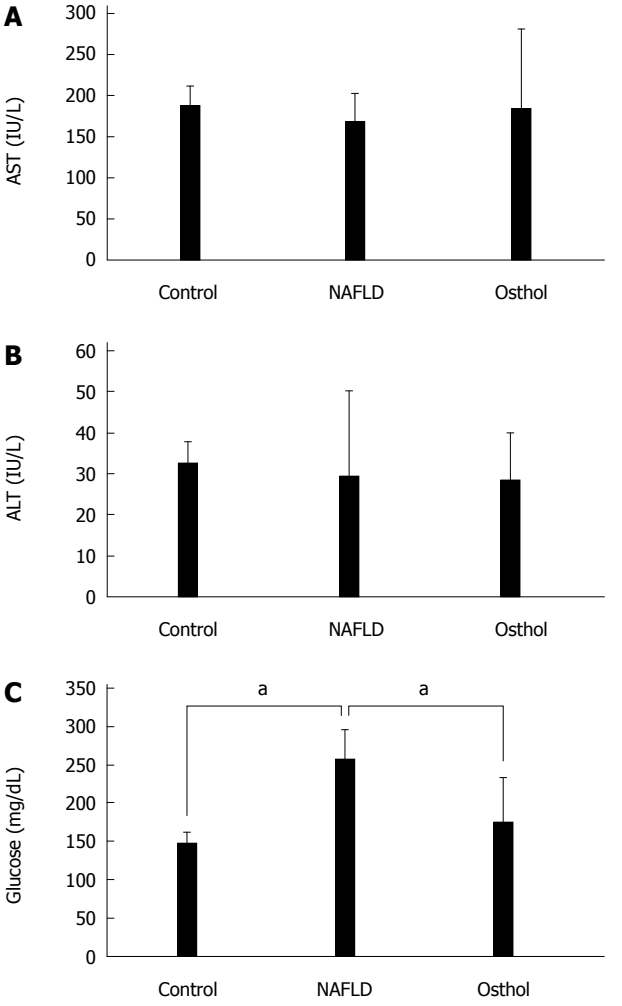

Figure 2 Effect of osthol on serum aspartate transaminase/alanine transaminase and glucose levels. Changes in serum ASLT/ALT of control, non-alcoholic fatty liver disease (NAFLD) and osthol groups (A and B). Osthol treatment decreased serum glucose levels (C). AST: Aspartate aminotransferase; ALT: Alanine aminotransferase. $^{a}P < 0.05$.

(score, 1), > 33%-66% (score, 2) and > 66% (score, 3); steatosis, 0-3; lobular inflammation, 0-4; portoperiportal activity, 0-4; and fibrosis, 0-4. For immunohistochemistry, the sections were stained with 4-hydroxynonenal (4-HNE) antibodies (Abcam, Cambridge, MA, USA) to assess lipid peroxidation. The macrophages were stained using F4/80 antibodies (Santa Cruz, CA, USA).

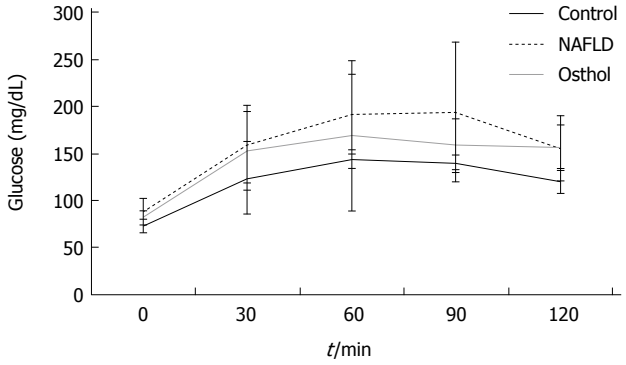

Figure 3 Changes in serum glucose levels following the oral glucose tolerance test. NAFLD: Non-alcoholic fatty liver disease.

PCR expression of SREBP1c, FAS, SCD-1, PPAR-α, CROT, IRS-1 and -2, and MCP-1

The total liver tissue RNA of each group was acquired using the Trizol Reagent (Invitrogen, United States). The RNA purity (1.9-2.0) was measured based on ration A260-280 with the Nano drop ND-2000 spectrophotometer (Thermo Fisher Scientific Inc., USA). The PCR had an incubation time of 10 min at 95 ℃ then 35 cycles (10 s at 95 ℃, 59 ℃, and 72 ℃ each) and 15 s at 65 ℃ for the final step. Image density was measured with Image J (http://rsb.info.nih.gov/ij/index.html). The primer sets used were as follows: GAPDH (Gene Bank ID: 24383) forward, 5'-TGC CAC TCA GAA GAC TGT GG-3'; reverse, 5'-TTC AGC TCT GGG ATG ACC TT-3'; SREBP1c (Gene Bank ID: 78968) forward, 5'-CGT TGT ACT GCA GCC ACA CT-3'; reverse, 5'-TGT GCT GTA AGA AGC GGA TG-3'; FAS (Gene Bank ID: 50671) forward, 5'-GAG TCT GTC TCC CGC TTG AC-3'; reverse, 5'-CCC TCC AGC ATG TAG ACC TT-3'; SCD-1 (Gene Bank ID: 246074) forward, 5'-ACC TTG CTC TGG GGG ATA TT-3'; reverse, 5'-GAT GAA GCA CAT GAG CAG GA-3'; PPAR-α (Gene Bank ID: 25747) forward, 5'-GAC AAG GCC TCA GGA TAC CA-3'; reverse, 5'-GTC TTC TCA GCC ATG CAC AA-3'; IRS-1 (Gene Bank ID: 25467) forward, 5'-ACA CAG CTG CAC AGA CCA AC-3'; reverse, 5'-CCC AAC TCA ACT CCA CCA CT-3'; IRS-2 (Gene Bank ID: 29376) forward, 5'-CAT CCA TGG CCT TCT CTC TC-3'; reverse, 5'-CCA TGA GAC TTA GCC GCT TC-3'; CROT (Gene Bank ID: 83842) forward, 5'-TCC GGA TGC TGT TTT CTA CC-3'; reverse 5'-GTT GCA TGT GGA CTG GTG TC-3'; ATF6 (Gene Bank ID: 304962) forward, 5'-CCC ACC AAA GGT CAG ACT GT-3'; reverse, 5'-

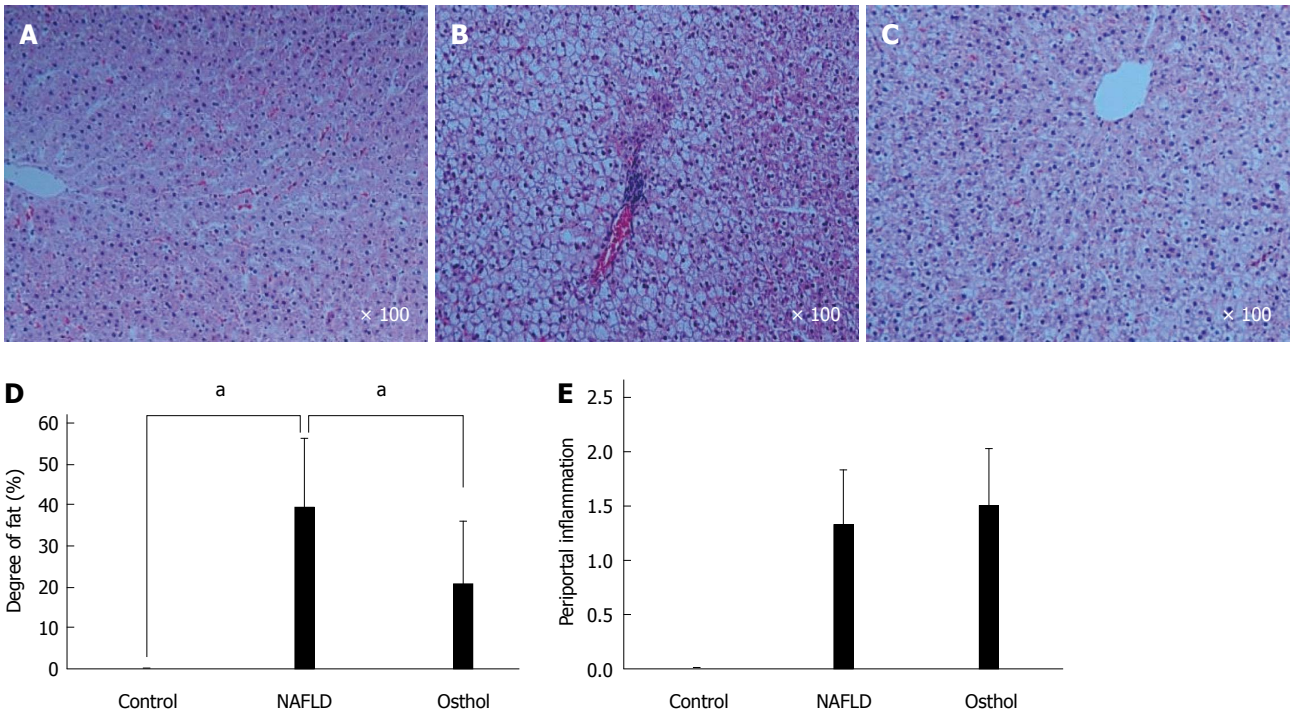

Figure 4 Effect of osthol on liver histology. Hematoxylin and eosin staining showing the difference in periportal inflammation and fat content between control, NAFLD and osthol groups (A-E). $^{a}P < 0.05$.

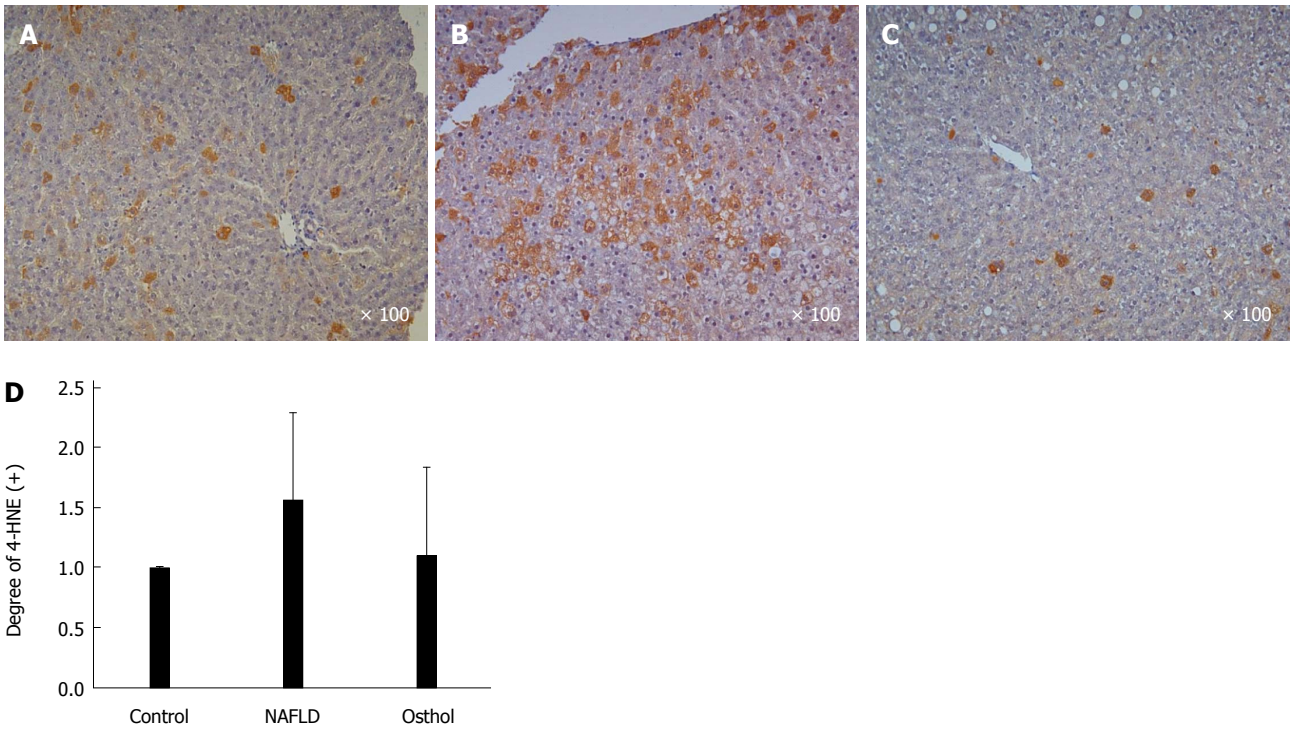

Figure 5 4-hydroxynonenal immunohistostaining comparing control, non-alcoholic fatty liver disease, and osthol groups (A-D). The NAFLD group shows increased 4-hydroxynonenal (4-HNE) immunohistostaining compared with the osthol group. NAFLD: Non-alcoholic fatty liver disease.

CTT GGG ACT TTG AGC CTC TG-3'; MCP1 (Gene Bank ID: 24770) forward, 5'-TAG CAT CCA CGT GCT GTC TC-3'; reverse, 5'-GCT TGA GGT GGT TGT GGA AA-3'.

Statistical analysis

All experiments were independently repeated 3 times. The values are expressed as mean ± standard deviation. Statistical analysis was performed using SPSS for Windows

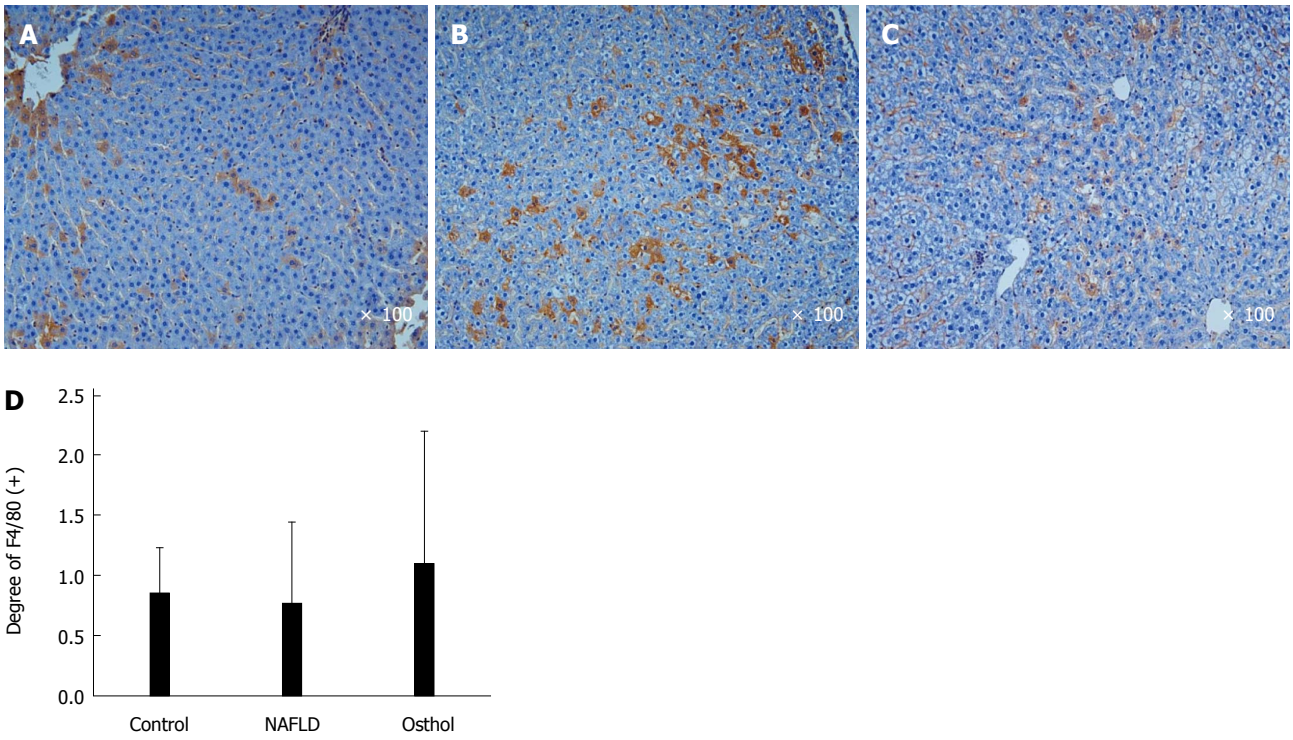

Figure 6 F4/80 immunohistostaining comparing control, non-alcoholic fatty liver disease, and osthol groups showing no significant difference between the groups (A-C). NAFLD: Non-alcoholic fatty liver disease.

version 18.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance was performed to compare the means of different values, and a *P*-value < 0.05 was considered significant.

RESULTS

Physical and biochemical parameters

The average body weight of both NAFLD and osthol groups were higher than in the control group. However, there was no statistically significant difference in body weights of NAFLD and osthol groups (695.4 ± 61.2 g *vs* 685.7 ± 86.62 g, $P = 0.78$) (Figure 1). Moreover, the serum AST and ALT levels among the 3 groups also did not show any statistically significant difference (Figure 2A and B). The fasting blood glucose levels of both NAFLD and osthol groups were higher than controls. However, compared with the NAFLD group, fasting blood glucose was lower in the osthol group (258.3 mg/dL *vs* 175.8 mg/dL, $P < 0.04$) (Figure 2C). Glucose intolerance was assessed using the OGTT. In NAFLD and osthol treatment groups, the area under the receiver operating characteristic curve for the osthol group was lower than that for the NAFLD group, but this difference was not statistically significant (Figure 3).

Hepatic fat content, intrahepatic inflammation, and fibrosis

On HE staining the osthol group showed a statistically significant ($P = 0.021$) decrease in hepatic fat content compared with the NAFLD (21% and 39.44% respectively). However, there was no difference in periportal inflammation and degree of intrahepatic fibrosis between the NAFLD and osthol groups (Figure 4A-E). 4-HNE immunohistochemistry was performed to evaluate the extent of lipid peroxidation. 4-HNE staining revealed that the osthol group had a decrease in lipid peroxidation compared with the NAFLD group, but the difference was not statistically significant (Figures 5 and 6).

SREBP1c, FAS, SCD-1, PPAR-α, and CROT expression

The transcription factor sterol regulatory element binding protein-1c (SREBP1c) regulates several lipogenic enzymes including acetyl-CoA carboxylase, pyruvate kinase, fatty acid synthase (FAS), and stearyl-CoA desaturase (SCD-1)[19-23]. SREBP1c, FAS and SCD-1 expression is increased in NAFLD[24,25]. We assessed the mRNA expressions of SREBP1c, FAS and SCD-1 to evaluate *de novo* intrahepatic fatty acid synthesis. SREBP1c expression increased in both NAFLD ($P < 0.0001$) and osthol groups compared with the control group. However, the expression of SREBP1c was lower in the osthol group compared with the NAFLD group ($P = 0.0059$) (Figure 7A). Similarly, the expression of both FAS and SCD-1 also increased in the osthol and NAFLD groups, but the expression was lower in the osthol compared with the NAFLD group ($P = 0.001$ and $P = 0.059$ respectively) (Figure 7B and C).

The mRNA expression of PPAR-α and carnitine octanoyltransferase (CROT) was assessed to evaluate intra-

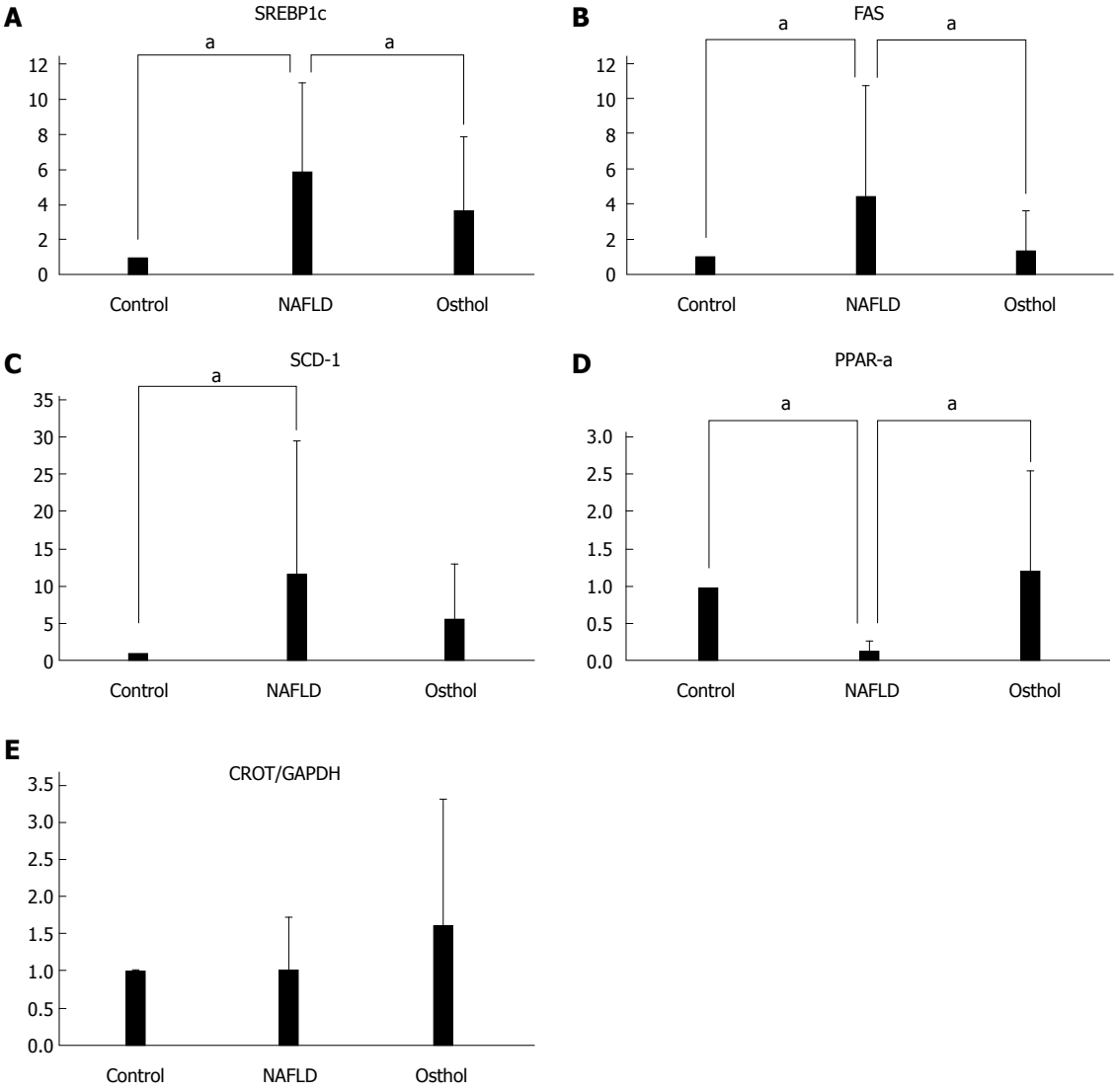

Figure 7 Polymerase chain reaction expressions of SREBP1c, FAS, SCD-1, PPAR-α and CROT. Compared with the NAFLD group, osthol treatment decreased SREBP1c, FAS, and SCD-1 expression (A-C), and increased PPAR-α and CROT expression (D and E). $^{a}P < 0.05$.

hepatic lipids metabolism. The PPARs transcriptionally regulate certain genes including PPAR-α and PPAR-γ, which in turn regulate lipid metabolizing enzymes[14]. In the osthol group, PPAR-α expression significantly increased compared with that in the NAFLD group ($P = 0.0147$) (Figure 7D). Similarly, mRNA expression of CROT, which controls the transfer of fatty acids to mitochondria for β-oxidation, also increased in the osthol group, although it was not significantly different from the NAFLD group (Figure 7E).

IRS-1, IRS-2, ATF6 and MCP-1 expressions

There was no statistically significant change in insulin receptor substrate-1 (IRS-1) and IRS-2 expressions (Figure 8A-B). Moreover, there was also no significant difference in expression of monocyte chemo-attractant protein-1 and activating transcription factor-6 (ATF6), an endoplasmic stress marker, between the osthol and NAFLD groups (Figure 8C and D).

DISCUSSION

NAFLD usually has a benign clinical course[26]; however, its inflammatory counterpart, the NASH can progress to chronic liver disease and fibrosis. Therefore, a reduction in hepatic inflammation and fibrosis is considered more important than a reduction in hepatic fat content. NASH is a "multi-hit" disease process which results from intrahepatic fat accumulation, increased oxidative stress, and abnormal hepatocyte adaptation[4]. The increased oxidative stress is an important risk factor leading to the progression of simple steatosis to steatohepatitis. In the current study, osthol treatment decreased the hepatic fat content mainly by decreasing *de novo* hepatic fat synthesis. Previous studies also reported that osthol treatment decreased hepatic fat content in NAFLD[15,27]. The decreased hepatic fat content is mainly due to improved insulin resistance and increased fat oxidation by PPAR-α activation.

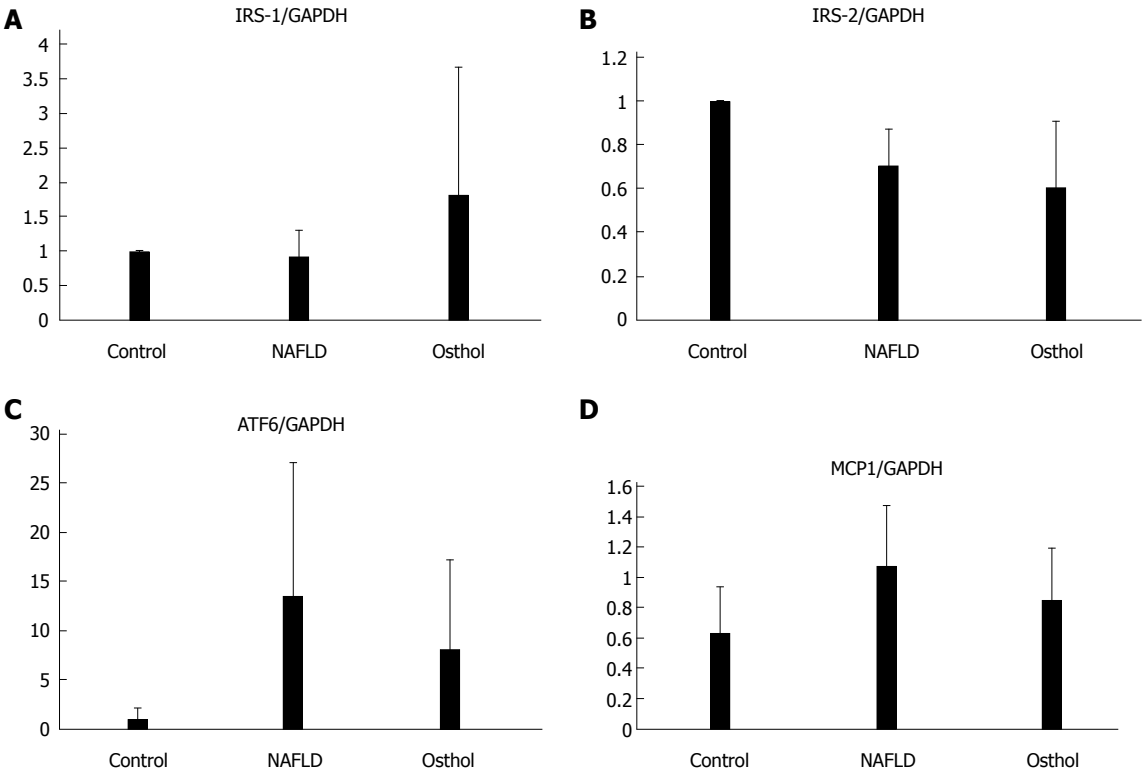

Figure 8 Polymerase chain reaction expressions of IRS-1, IRS-2, ATF6 and MCP-1 (A-D) showing no statistically significant differences between the groups.

Qi *et al*[13] reported that osthol treatment decreased HOMA-IR in NAFLD. In our study, osthol treatment lowered fasting blood glucose levels; however, there was no significant difference in OGTT between osthol and NAFLD groups, suggesting that osthol treatment did not affect the hepatic transcription factors involved in the insulin signaling pathway. SREBP1c is a key molecule in triglyceride synthesis. SCD-1 acts downstream of SREBP1 and FAS in the triglyceride synthesis pathway. In the current study, osthol treatment significantly decreased SREBP1c, FAS and SCD-1 expression. Moreover, compared with the NAFLD group, osthol treatment also increased PPAR-α and CROT expression, suggesting that osthol increases fatty acid oxidation. ATF6, an ER stress marker, was also decreased in the osthol group, but this was not statistically significant.

Zhang *et al*[16] reported a decrease in tumor necrosis factor-α, SOD and malondialdehyde following osthol administration. Moreover, previous studies also reported the possibility of decreased intrahepatic inflammation because of a reduction in surrogate markers of inflammation. However, none of the previous studies showed that osthol could attenuate intrahepatic inflammation as well as hepatic apoptosis. In our study, osthol administration did not decrease hepatic inflammation, fibrosis, and aminotransferases. Moreover, the MCP-1 and Kupffer cells, which have a crucial role in NASH development, were also not changed (Figures 6 and 8D, respectively). Furthermore, only Sun *et al*[27] showed that osthol decreased intrahepatic oxidative stress using an alcoholic fatty liver model. However, the oxidative stress has a different role in NAFLD and alcoholic fatty liver disease; therefore, these results cannot be compared directly.

Our study had the following limitations: First due to small size of the study, we used blood glucose, OGTT test, and total IRS to assess insulin resistance; however, the euglycemic clamp test is the gold standard for measuring insulin resistance[28]. Second, to induce liver inflammation and fibrosis, an HF diet was applied for 14 wk. However, to further evaluate the efficacy of osthol on liver inflammation and fibrosis, the duration of the HF diet should be extended, or genetically modified animals along with diet model should be used. Third, we did not evaluate the decrease in hepatic fat synthesis caused by decreased SREBP1c due to hepatocyte adaptation or apoptosis. Further studies are needed to evaluate the efficacy of osthol in improving liver inflammation and mitochondrial β-oxidation.

In conclusion, osthol administration for 6 wk decreased *de novo* hepatic fat synthesis and improved fatty liver by decreasing SREBP1c and increasing PPAR activation; however, the osthol treatment did not attenuate intrahepatic inflammation and fibrosis. Osthol may be used as a potential therapeutic agent to prevent NAFLD progression as a result of its ability to decrease *de novo* hepatic fat synthesis and to increase fatty acid oxidation.

COMMENTS

Background

The "two hit" theory is the widely accepted theory to explain non-alcoholic fatty liver disease pathophysiology. The first hit comprises accelerated lipidsaccumula-

tion, while the second hit comprises increased lipid oxidation in the liver. Osthol, a coumarin compound, possesses anti-inflammatory and fat oxidizing properties. The authors aimed to evaluate the effects of osthol on hepatic fat content.

Research frontiers

Osthol has anti-inflammatory and fat oxidization effects. In this study, the authors to simultaneously evaluated the effects of osthol on fat oxidation, inflammation, fibrosis and insulin resistance pathways.

Innovations and breakthroughs

The fat oxidizing effects of osthol have already been well documented. However, interestingly, none of previous studies evaluated the effect of osthol on liver inflammation and fibrosis in addition to the fat oxidizing effects. This is the first study to evaluate simultaneously the effects of osthol on inflammation, fibrosis, fat oxidation, and insulin resistance.

Applications

By understanding the mechanism of osthol effects on various pathways involved in NAFLD pathology, this study provides useful information for future studies and also highlights the potential use of osthol to slow progression of NAFLD.

Terminology

Cnidium monnieri fruits are used in traditional Chinese medicine. Osthol is the active ingredient of Cnidium monnieri extracts.

Peer review

In this study Ho Hyun Nam *et al* investigated the effects of osthol on intrahepatic fat synthesis, β-oxidation, inflammation and insulin resistance by multifaceted-analysis in a group of 30 rats randomly divided into control, NASH and osthol groups.

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ORIGINAL ARTICLE

Comparison of Abbott and Da-an real-time PCR for quantitating serum HBV DNA

Ning Qiu, Rui Li, Jian-Guo Yu, Wen Yang, Wei Zhang, Yong An, Tong Li, Xue-En Liu, Hui Zhuang

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Abstract

AIM: To compare the performance of the Da-an real-time hepatitis B virus (HBV) DNA assay and Abbott RealTime HBV assay.

METHODS: HBV DNA standards as well as a total of 180 clinical serum samples from patients with chronic hepatitis B were measured using the Abbott and Da-an real-time polymerase chain reaction (PCR) assays. Correlation and Bland-Altman plot analysis was used to compare the performance of the Abbott and Da-an assays. The HBV DNA levels were logarithmically transformed for analysis. All statistical analyses were performed using SPSS for Windows version 18.0. The correlation between the two assays was analyzed by Pearson's correlation and linear regression. The Bland-Altman plots were used for the analysis of agreement between the two assays. A P value of < 0.05 was considered statistically significant.

RESULTS: The HBV DNA values measured by the Abbott or Da-an assay were significantly correlated with the expected values of HBV DNA standards ($r = 0.999$, for Abbott; $r = 0.987$, for Da-an, $P < 0.001$). A Bland-Altman plot showed good agreement between these two assays in detecting HBV DNA standards. Among the 180 clinical serum samples, 126 were quantifiable by both assays. Fifty-two samples were detectable by the Abbott assay but below the detection limit of the Da-an assay. Moreover, HBV DNA levels measured by the Abbott assay were significantly higher than those of the Da-an assay (6.23 ± 1.76 log IU/mL *vs* 5.46 ± 1.55 log IU/mL, $P < 0.001$). A positive correlation was observed between HBV DNA concentrations determined by the two assays in 126 paired samples ($r = 0.648$, $P < 0.001$). One hundred and fifteen of 126 (91.3%) specimens tested with both assays were within mean difference ± 1.96 SD of HBV DNA levels.

CONCLUSION: The Da-an assay presented lower sensitivity and a narrower linear range as compared to the Abbott assay, suggesting the need to be improved.

Key words: Hepatitis B virus; Hepatitis B virus DNA quantitation; Real-time polymerase chain reaction; Chronic hepatitis B; Antiviral therapy

Core tip: The hepatitis B virus (HBV) DNA values measured by the Abbott or Da-an real-time polymerase chain reaction assay were significantly correlated with the expected values of HBV DNA standards. A Bland-Altman plot showed good agreement between the assays. For clinical evaluation, HBV DNA levels derived

from the Abbott assay were significantly higher than those of the Da-an assay.

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INTRODUCTION

An estimated 350 million people worldwide are chronically infected with hepatitis B virus (HBV) and about one-third of these live in China[1]. The spectrum of disease and natural history of chronic HBV infection are diverse and variable, ranging from an inactive carrier state to progressive chronic hepatitis B (CHB), which may evolve to cirrhosis and hepatocellular carcinoma (HCC)[2]. Longitudinal studies of untreated patients with CHB indicate that the 5-year cumulative incidence of developing cirrhosis ranges from 8% to 20% following diagnosis. The 5-year cumulative incidence of hepatic decompensation is approximately 20% for untreated patients with compensated cirrhosis. Untreated patients with decompensated cirrhosis have a poor prognosis, with only a 14%-35% probability of survival at 5 years. The annual incidence of HBV-related HCC in patients with cirrhosis ranges from 2% to 5%[2]. Antiviral therapy against HBV is an important measure for preventing and delaying progression of the disease from CHB to cirrhosis, end-stage liver disease, HCC, and death[3]. During therapy, quantification of HBV DNA plays a crucial role in the management of CHB by allowing criteria to be established for determining patient eligibility for antiviral therapy, monitoring treatment response, and identifying the emergence of resistance in order to adapt therapy[4].

Sensitive and accurate quantification of HBV DNA is necessary to monitor patients with CHB who are receiving antiviral therapy[5]. An assay with good sensitivity and accurate detection for quantitating HBV DNA level will contribute to optimal monitoring of antiviral therapy, early confirmation of drug resistance, and timely treatment adaption. Measurement of viral load is mostly accomplished by detection of HBV DNA in serum or plasma with nucleic acid amplification or signal amplification technologies. Real-time polymerase chain reaction (PCR), one of the target (HBV DNA) amplification techniques, has been developed with more sensitivity and broader dynamic range when compared to signal amplification technique[4]. Several real-time PCR-based commercially available tests for the quantitation of HBV DNA in serum or plasma specimens are routinely used in diagnostic laboratories in China. The Da-an real-time HBV DNA assay (Da-an Gene Co. Ltd, Sun Yat-Sen University, Guangdong, China) is one of them. However, internationally ubiquitous real-time assays, including the COBAS TaqMan HBV test (Roche Molecular Diagnostics, Pleasonton, CA, United States), Abbott RealTime HBV assay (Abbott Molecular, Des Plaines, IL, United States), and the Artus RealART HBV LC PCR kit (QIAGEN, Hamburg, Germany) are rarely used in clinical practice in China due to their high cost.

The present comparative study was carried out to explore the difference and correlation between a domestic assay (Da-an assay) and an internationally accepted assay (the Abbott assay). The performance of the two assays was evaluated, and the correlation and agreement between them were analyzed.

MATERIALS AND METHODS

Clinical samples

A total of 48 patients with chronic hepatitis B aged 17-65 years (35 male and 13 female) was enrolled in this study. These patients had received adefovir as antiviral therapy at the hospital since 2010. A hundred and eighty serum samples were obtained at week 0, 12, 24, 36 and 48 during antiviral treatment. All samples were stored in the laboratory at -70 ℃.

This study was approved by the Ethics Committee of Peking University Health Science Center in accordance with the Helsinki Declaration. Informed consent was obtained from each patient.

Standards (reference sera)

A panel of reference sera for quantitating HBV DNA was provided by the Chinese National Institutes for Food and Drug Control. The HBV DNA reference panel was used to evaluate the sensitivity of the assays, which consisted of eight negative controls (N1-8), nine positive controls (P1-9), and seven sensitivity standards (L0-6). The sensitivity standards L0-6 were made from a dilution series of a single serum sample with HBV-marker-negative serum. This panel of reference sera was standardized by the WHO international standard for HBV DNA (NIBSC10/264) and was measured with several internationally accepted commercial assays for quantitating HBV DNA. The range of concentrations of the sensitivity sera L0-6 are listed in Table 1, and the logarithmic mean concentrations of standards L0-6 were 2.04, 3.64, 4.72, 5.67, 6.65, 7.62 and 8.34 log IU/mL, respectively. The HBV genotype of the sensitivity standard (L0-6) is genotype B. The panel was produced by the Chinese National Institutes for Food and Drug Control and data were provided by the package insert instruction of the panel. The panel was stored at -70 ℃.

Abbott realtime HBV assay

The serum samples were processed using m2000sp, an automatic nucleic acid extraction apparatus that uses a magnetic microparticle-based principal for DNA purification. Amplification was performed on an Abbott m2000rt real-time instrument. An initial serum volume

Table 1 Inter-assay analysis of hepatitis B virus DNA levels of reference sera measured by the Abbott and Da-an assays

Expected concentration of HBV DNA standard (log IU/mL)	Abbott assay (log IU/mL) (mean ± SD)	CV	Da-an assay (log IU/mL) (mean ± SD)	CV
L0: 8.34 (7.89-8.79)	8.64 ± 0.07	0.8%	8.13 ± 0.17	2.0%
L1: 7.62 (7.17-8.07)	7.91 ± 0.04	0.5%	7.65 ± 0.10	1.3%
L2: 6.65 (6.20-7.10)	6.89 ± 0.05	0.7%	6.79 ± 0.01	0.2%
L3: 5.67 (5.22-6.12)	5.89 ± 0.01	0.2%	6.53 ± 0.65	10.0%
L4: 4.72 (4.26-5.17)	4.92 ± 0.08	1.7%	5.27 ± 0.21	3.9%
L5: 3.64 (3.18-4.09)	3.85 ± 0.24	1.1%	3.96 ± 0.04	1.0%
L6: 2.04 (1.59-2.49)	2.33 ± 0.07	3.0%	2.19 ± 0.01	0.4%

CV: Coefficients of variation; HBV: Hepatitis B virus.

of 200 μL was used for the nucleic acid extraction, and the final elution volume was 70 μL. Fifty microliters of elute was used as an amplification template. 15 IU/mL (1.18 log IU/mL, for 200-μL sample) of the limit of detection (LOD) in the Abbott assay was determined by testing dilutions of the WHO International Standard for HBV DNA (NIBSC97/746). The upper limit of quantitation for the Abbott assay was 10^9 IU/mL and the lower limit of quantitation was equivalent to LOD (15 IU/mL for 0.2-mL sample). A specimen with a result of "not detected" was defined to be negative and a result of "< 15 IU/mL" was presumed to be under the limit of detection. Samples with HBV DNA levels above the upper limit (10^9 IU/mL) were diluted and remeasured at appropriate concentrations.

The Da-an real-time PCR HBV DNA assay

The procedure of the Da-an real-time PCR HBV DNA assay (catalog number: DA-D051) consisted of extracting nucleic acid manually followed by DNA quantitation with real-time PCR. Nucleic acid extraction in detail: (1) serum sample with a starting volume of 100 μL was prepared in an Eppendorf tube, then 100 μL concentrated solution was added and mixed by pulse vortexing for 5 s; (2) centrifugation at 12000 rpm for 10 min; (3) discard the upper liquid, then add the extraction reagent 20 μL to the precipitation and mix by pulse vortexing for 5-10 s, centrifuge for 5 s; (4) the tube was placed in a water bath at 100 ℃ for 10 min; and (5) centrifugation at 12000 rpm for 5 min, and the final 20 μL of extracted nucleic acid was used as the template. Two microliters of the purified nucleic acid was added to the real-time PCR mixture (for a final volume of 45 μL) for amplifying the target HBV surface gene. The TaqMan probe was used in this real-time PCR amplification system, which was performed on a LightCycler 480 system (Roche) by incubating the reaction mixture at 93 ℃ for 2 min, followed by 40 cycles of PCR amplification at 93 ℃ for 5 s and 57 ℃ for 45 s. The dynamic range of the Da-an assay was 10^3-10^8 IU/mL for the first-generation products. The result of HBV DNA level "< 3 log IU/mL" was also reported and considered as an inaccurate result because it was beyond the linear (dynamic) range. It was reported as negative while the C_T (the cycle of threshold) values of the samples were shown as 40, 0 or blank.

HBV genotyping

HBV DNA was extracted from 200 μL serum using a QIAamp DNA Blood Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Nested PCR was used to amplify the entire reverse transcriptase (RT) region of HBV. The PCR conditions and the sequences of the nested PCR primers were the same as described by Yang *et al*[6]. The product of PCR with approximately 1195 base pairs was visualized on 1% agarose gel, purified, and sequenced commercially (Shanghai Invitrogen Biotechnology Co. Ltd., Shanghai, China)

HBV genotyping was determined using the NCBI Viral Genotyping Tool (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi) and phylogenetic analysis with MEGA 4.0 software.

Statistical analysis

The HBV DNA levels were logarithmically transformed for analysis. All statistical analyses were performed using SPSS for Windows version 18.0 (SPSS, Chicago, IL, United States). The correlation between the two assays was analyzed by Pearson's correlation and linear regression. The Bland-Altman plots were used for the analysis of agreement between the two assays. A P value of < 0.05 was considered statistically significant.

RESULTS

Analytical evaluation

A total of eight negative references and nine positive references from the HBV DNA standard panel sera were correctly detected by the Abbott or Da-an assay. For the seven sensitivity references, as shown in Figure 1, HBV DNA values measured by the Abbott or Da-an assays were significantly correlated with the expected values of HBV DNA standards ($r = 0.999$, $P < 0.01$, for Abbott; $r = 0.987$, $P < 0.01$, for Da-an, respectively). Furthermore, good agreement between the results of the two assays for detecting HBV DNA standards was observed by the Bland-Altman analysis (Figure 2). All of the difference values of the paired viral loads were within the range of mean difference ± 1.96 SD (log IU/mL). The mean value of differences of the paired viral loads, 95% confidence interval, and maximal difference were -0.01 log IU/mL, (-0.77, 0.75) log IU/mL, and 0.64 log IU/mL, respectively, thus indicating that there was no significant difference between these two assays.

The reference sera L0-6 were tested three times with the Abbott and Da-an assay over a period of 3 d. All of the means of quantitative results obtained with the Abbott assay were within the range of the expected values with the inter-assay variation ranging from 0.2% to 3.0%. For HBV DNA levels detected with the Da-an assay, two of the seven mean values were out of the reference ranges with the inter-assay variation ranging from 0.2% to 10% (Table 1).

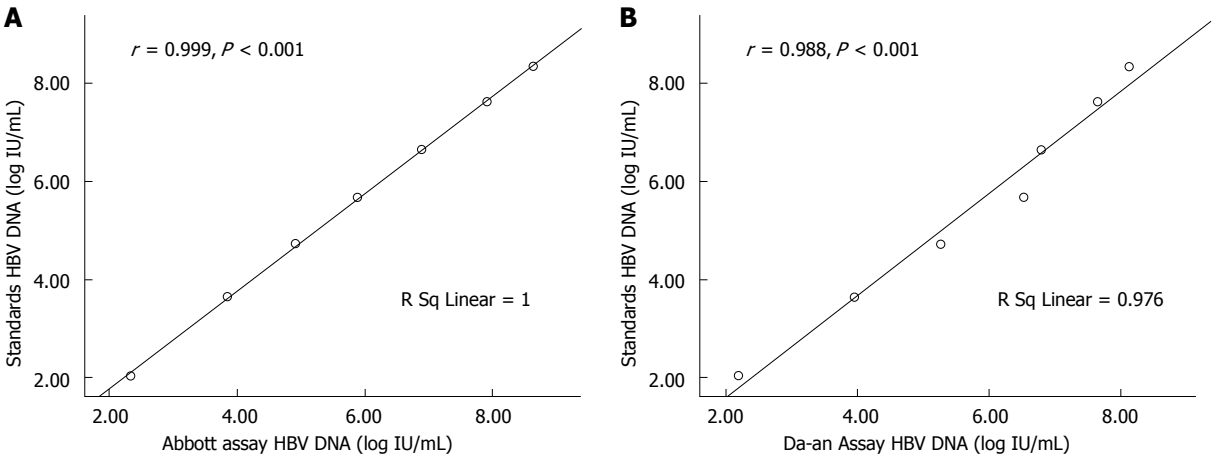

Figure 1 Correlation analysis. Correlation analysis between the expected hepatitis B virus (HBV) DNA concentration (log IU/mL) in the sensitivity references (standards) and the corresponding test results in the Abbott (A) and Da-an (B) assays. Each point represents the mean log IU/mL of three data points tested over 3 d.

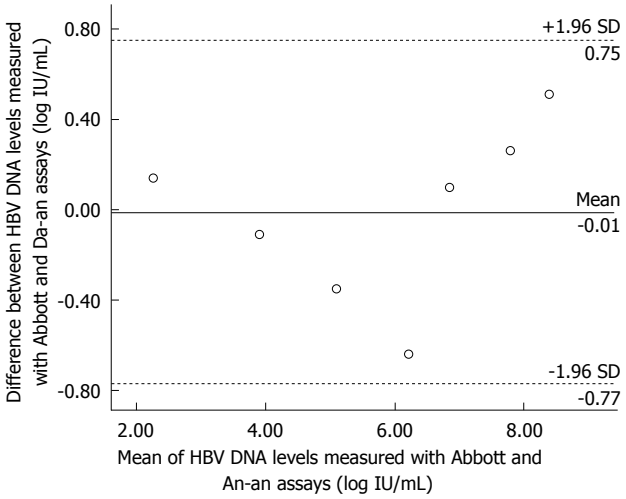

Figure 2 Bland-Altman analysis. Bland-Altman analysis of hepatitis B virus (HBV) DNA level measured with the Abbott and Da-an assays in seven sensitivity references of HBV DNA standards. The difference between the Abbott and Da-an measurements is plotted as a function of the mean of the two values. The area between the dashed lines corresponds to the mean difference ± 1.96 SD.

Clinical evaluation

All of the 48 CHB patients were infected with HBV genotype C. This genotype dispersal conformed to the HBV genotype distribution profile in Northern China[3,6]. Among the 180 clinical serum samples, 126 had a detected value by the Da-an assay while 178 were quantitated by the Abbott assay. Comparison of HBV DNA levels measured by these two assays is shown in Table 2. The only discrepancy with the results is that one sample was below the detection limit of the Abbott assay, but was detectable when quantitated by the Da-an assay. This serum sample was taken at week 36 of adefovir therapy from patient No. 1. The HBV DNA levels of this patient at week 24, 36 and 48 of treatment were 4.77, < 1.18 IU/ and < 1.18 IU/mL, respectively, for the Abbott test, and 3.23, 4.99 and < 3 IU/mL for the Da-an test. It could be deduced that a false-positive result with the Da-an assay may have occurred.

Table 2 Comparison between hepatitis B virus DNA levels of 180 serum samples from 48 patients with chronic hepatitis B measured with the Abbott and Da-an real-time polymerase chain reaction assays

Abbott RealTime assay result (log IU/mL)	Da-an real-time HBV DNA assay result (log IU/mL)			
	⩾ 8.00	⩾ 3.00-< 8.00	< 3.00 (negative)	Total
> 9.00	0	3	0	3
⩾ 1.18-⩽ 9.00	3	120	52	175
< 1.18	0	1	0	1
Negative	0	0	1	1
Total	3	124	53	180

HBV: Hepatitis B virus.

The paired HBV DNA levels of the 126 samples detectable by both assays were analyzed. The mean logarithmic level of HBV DNA quantitated by the Abbott assay was significantly higher than that by the Da-an assay (6.23 ± 1.76 log IU/mL *vs* 5.46 ± 1.55 log IU/mL, $P < 0.01$). As shown in Figure 3, correlation analysis showed that a significantly positive correlation was obtained between HBV DNA concentrations ($r = 0.648$, $P < 0.01$). In addition, samples were divided into two groups (⩾ 5.00 log IU/mL and < 5.00 log IU/mL) according to the viral load detected by the Abbott assay. There was a positive correlation between the paired HBV DNA levels in the group with a higher viral load ($r = 0.665$, $P < 0.01$), but no correlation in the group with the lower viral load ($r = 0.321$, $P = 0.073$).

The agreement analysis for the HBV DNA levels in 126 clinical samples tested by these two assays was shown in Figure 4. The largest differences in the HBV DNA values of the paired samples were located in the range of mean difference ± 1.96 SD (115/126). The proportions of specimens with < 1 log, 1-2 log, and > 2 log difference of HBV DNA levels between the assays were 44.5% (56/126), 42.0% (53/126) and 13.5% (17/126), respectively. The mean difference value of the paired viral

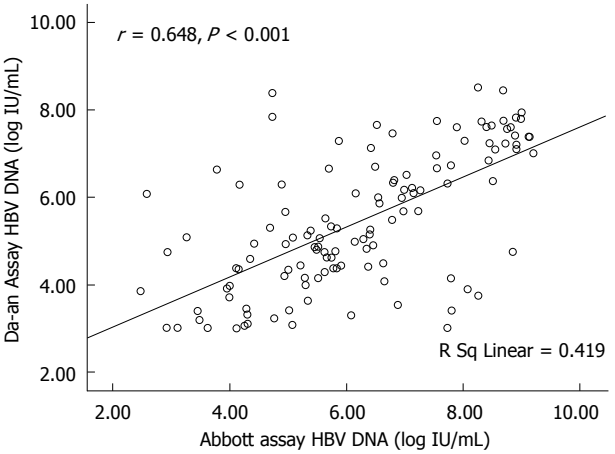

Figure 3 Correlation analysis. Correlation analysis between hepatitis B virus (HBV) DNA levels tested with the Abbott and Da-an assays in 126 paired serum samples of patients with chronic hepatitis B. HBV DNA levels (log IU/mL) measured with Da-an assay were plotted against viral load (log IU/mL) determined with the Abbott assay.

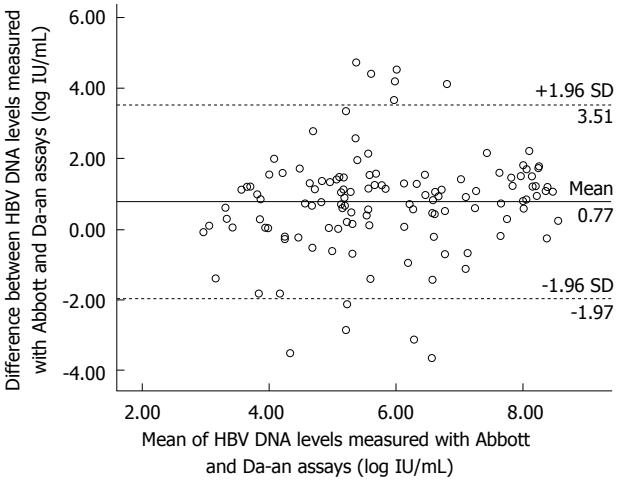

Figure 4 Bland-Altman analysis. Bland-Altman analysis of hepatitis B virus (HBV) DNA level measured with the Abbott and Da-an assays in 126 clinical serum samples from patients with chronic hepatitis B. The difference between the Abbott and Da-an measurements is plotted as a function of the mean of the two values. The area between the dashed lines corresponds to the mean difference ± 1.96 SD.

loads, 95% confidence interval, and maximal difference were 0.77, (-1.97, 3.51) and 4.72 log IU/mL, respectively.

Fifty-two samples were detectable by the Abbott assay but below the detection limit of the Da-an assay. HBV DNA levels of these 52 samples ranged from 1.26 to 8.39 log IU/mL. The distribution of HBV DNA concentrations were 1.18-2 log IU/mL, 12 samples; 2-3 log IU/mL, eight samples; 3-4 log IU/mL, nine samples; 4-5 log IU/mL, four samples; 5-6 log IU/mL, three samples; 6-7 log IU/mL, 10 samples; 7-8 log IU/mL, four samples; and 8-9 log IU/mL, two samples; respectively.

At the baseline of adefovir dipivoxil therapy, the entire RT region of HBV in serum samples from 48 CHB patients was amplified and sequenced commercially. Lamivudine resistants (L180M, M204V and M204I) were found in two of the 48 patients (Nos. 23 and 41). The HBV DNA levels of patient No. 41 during treatment follow-up were 8.26 log, 3.91, 3.63, 3.91 and 2.57 log IU/mL for the Abbott assay; 6.32 log, < 3, < 3, < 3 and < 3 log IU/mL for the Da-an assay. The same trend was observed in another patient. Based on the dynamic changes of HBV DNA levels, adefovir dipivoxil was an effective antiviral drug for both of the lamivudine-resistant patients. At the same time, the differences of HBV DNA levels measured by both assays were also observed.

DISCUSSION

The three major liver societies, the American Association for the Study of Liver Diseases[7], the European Association for the study of the Liver[2], and the Asia-Pacific Association for the Study of the Liver[8], have all issued guidelines for the management of CHB that specify certain HBV DNA thresholds to determine which patients are candidates for antiviral treatment. Viral load measurement is used not only in the diagnosis of HBV but also in the monitoring of patients for effective antiviral treatment, although the respective guidelines differ in the recommended intervals for such testing. In China, HBV DNA levels should be detected every 3 mo in patients with CHB during antiviral therapy[3]. The quantitation of viral load is a routinely performed molecular test in clinical laboratories. The Da-an real-time HBV DNA test, produced domestically and approved by the State Food and Drug Administration, China for *in vitro* diagnostic use, is widely used in China. Therefore, it is necessary to compare the domestic assay to the international standard assay.

The real-time PCR-based commercial assays for HBV DNA quantitation used in clinical practice have been available worldwide for several years. There are some differences in sensitivity, specificity, dynamic range, and reproducibility among the assays. Based on our results, the Abbott RealTime HBV assay has a higher sensitivity as compared with the Da-an real-time HBV DNA assay (15 IU for 0.2 mL sample *vs* 1000 IU for 0.2 mL), and the dynamic range of the Abbott assay is also broader than that of Da-an assays (1.18-9.0 log IU/mL *vs* 3-8 log IU/mL). Among the 180 clinical serum samples, 126 were detected by both assays; 52 samples with viral load below the detection limit of the Da-an assay were detectable by the Abbott assay; one sample was below the detection limit for the Abbott assay but detectable by the Da-an assay; and one sample was negative for both assays. Numerous factors affect the accuracy of quantifying HBV DNA, such as sample volumes used for HBV DNA isolation, enzyme inhibitors in samples, different methods for extracting nucleic acid, and diversity in primers and fluorescent markers for real-time PCR. Several reasons for differences in sensitivity, the linear range and other differences between the two assays are analyzed in detail as follow.

The first difference between these two assays is that

they use different methods to extract nucleic acid. A boiling method applied in the Da-an assay could affect the purity of nucleic acids and the efficiency of HBV DNA isolated. In addition, the manual specimen preparation is labor-intensive and can cause run-to-run variability and specimen-to-specimen contamination[9]. A false-positive result with the Da-an assay may be due to sample-to-sample contamination. For the Abbott assay, HBV DNA is extracted from serum samples by the m2000sp, an automated sample preparation system designed to use magnetic microparticle-based reagents for the purification of nucleic acids from samples. One of the major advantages of automating the HBV DNA extraction is the ability to provide a standardized process among the samples. At the same time, the automated sample preparation system (m2000sp) combined with the m2000rt analyzer significantly reduces hand-on work time and labor intensity while reducing the risk of contamination and human error[10].

Furthermore, factors include the differences in sample volumes, final elution volumes, template volumes, and PCR volumes: *i.e.*, 100, 20, 2 and 45 μL for the Da-an assay, and 200, 70, 50 and 100 μL for the Abbott assay, respectively. Besides the sample and PCR volumes, the difference in the ratio of the final elution volume over the template volume also matters (70/50 μL for the Abbott and 20/2 μL for the Da-an), which means the lower the ratio of elution/template volume the higher HBV DNA concentration in the final PCR mixture. Thus a higher concentration of DNA template could enhance the detection rate. The effects of the sample volume and HBV DNA concentration in the template on the sensitivity of HBV DNA detection were also reported in a previous study[11].

Another concern is the target regions for PCR amplification. The Abbott assay selected the highly conserved region in the S gene of the HBV genome as the target region, which located in the N-terminal third of the S gene ensuring that the assay is not affected by YMDD mutants, HBsAg escape mutants, or drug-resistant mutants, because this region is essential for the assembly and secretion of subviral particles, and tolerates minor structural changes. Therefore, the Abbott assay provides for the detection of genotypes A-H. The Da-an assay also selected a relatively conserved target region within the S gene of the HBV genome. However, the manufacturer did not specify the range of genotypes for the assay.

As a final point, the effect of the internal control applied in monitoring PCR amplification was demonstrated. One prerequisite for the PCR-based quantitative approach is to avoid PCR inhibitory substances, such as hemoglobin or heparin, in clinical samples. There are no external controls that can adequately control for these conditions, thus, false-negative test results can be generated[12,13]. In the Abbott assay, a DNA sequence unrelated to the HBV target sequence is introduced into the sample preparation procedure and processed with the calibrators, controls, and specimens. It serves as an internal control to compensate for the differences in DNA extraction efficiency between specimens and possible PCR inhibition in the reaction mixtures[14], which further controls for target isolation and amplification. The Da-an assay does not include such an internal control, thus, it does not control for the loss of nucleic acid during the process of extraction, causing suboptimal amplification or false-negative results.

We showed a strong correlation and good agreement between HBV DNA levels quantitated with the Abbott and Da-an assays in HBV DNA standards but not in clinical samples. Especially, 52 serum samples were detected by the Abbott assay but not by the Da-an assay. The reason may be that the Da-an assay could perform relatively well when testing the HBV DNA standards whose genotype is B. For all clinical samples with genotype C, the Da-an assay performed poorly. The Abbott assay provides for detection of genotype A-H. However, the Da-an assay did not declare that. It is possible that the Da-an assay could perform well, testing some genotypes of HBV but poorly when testing other genotypes. In addition, sequences of HBV DNA may change during antiviral therapy. The Abbott assay selected the highly conserved region in the S gene of the HBV genome as the target region and ensured that the assay is not affected by YMDD mutants, HBsAg escape mutants, or drug-resistant mutants. The Da-an assay also selected a relatively conserved target region within the S gene but did not exclude the impact of HBV DNA mutation. Zheng *et al*[15] reported that 200 serum samples were measured by the three real-time PCR reagents. Six out of 200 serum samples were underestimated or undetected by the Da-an assay. The sequence of the fluorescence probe binding region (FPBR) in HBV DNA genome of six serum samples was determined and compared with the sequence of HBV wild type. The mutations of the FPBR sequence were found and clarified that the mutations affected the measurement of HBV DNA.

Based on the above analysis, the manufacturer producing the Da-an real-time PCR HBV assay should improve their molecular technique, increase the sensitivity, and extend the dynamic range of the assay. As the related guidelines for managing chronic hepatitis B mention, the current standard of care is to adapt antiviral therapy in patients with drug resistance as early as possible, namely, at the time of viral breakthrough, which is defined by an increase in HBV DNA level by 1 log IU/mL compared to the nadir value[2,7,8,16]. It is important to detect lower viral load accurately, especially in viral breakthrough occurring when HBV DNA levels change from 10 to 100 IU/mL. The Abbott assay, with a sensitivity of 15 IU/mL for 200-μL serum samples, will be adequate for the detection of viral breakthrough at an early stage and allow for the rapid addition of a rescue therapy before clinical breakthrough[17]. An assay with good sensitivity and wide detection range for quantitating HBV DNA concentration is a crucial measurement to allow optimal monitoring of antiviral therapy and timely treatment adaption. The Da-an assay is not suitable for this clinical applica-

tion because it has inadequate sensitivity. Therefore, it is urgent to improve the quality of the Da-an assay for the manufacturer.

Although the Abbott assay has a higher sensitivity (15 IU *vs* 1000 IU), shorter assay time (4 h *vs* 8 h), and wider dynamic range (1.18-9.00 log IU/mL *vs* 3-8 log IU/mL) as compared with the Da-an assay, the costs of the Abbott assay are extremely high (approximately 50 US dollars/Abbott test *vs* 7 US dollars/Da-an test), which limits their routine use in clinical molecular laboratories in China. With respect to the Da-an assay, more improvements, including an automatic nucleic acid extraction apparatus and introducing an internal control, are needed for clinical practitioners. The disease burden of CHB is heavy in many developing countries. The patients with CHB receiving antiviral treatment are increasing annually. The domestic assays for quantitating HBV DNA were widely used not only in China but also in other developing countries. This comparative study would be helpful for manufacturers who have produced the products for HBV DNA quantitation in their countries.

In this study, the performance of the Da-an real-time HBV DNA assay and the Abbott RealTime HBV assay for quantitating HBV DNA levels were evaluated and compared. A strong correlation and good agreement between HBV DNA levels quantitated with the Abbott and Da-an assays was observed for testing the HBV DNA standard panel but not for clinical samples. As compared with the Abbott assay, the Da-an assay had lower sensitivity and a narrower linear range that needs further improvement.

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COMMENTS

Background

Sensitive and accurate quantification of hepatitis B virus (HBV) DNA is important to monitor patients with chronic hepatitis B during antiviral therapy. The domestic assays for quantitating HBV DNA are widely used in China and other developing countries. The comparative study was conducted to compare the performance of domestic and internationally accepted assays, to contribute valuable research data that would be helpful for improving the domestic assays in developing countries.

Innovations and breakthroughs

This is believed to be the first report on the comparison of the Abbott and Da-an real-time polymerase chain reaction assays for quantitating HBV DNA in serum. A strong correlation and good agreement between HBV DNA levels quantitated with the Abbott and Da-an assays was observed for testing the HBV DNA standard panel but not for clinical samples. The Da-an assay presented lower sensitivity and a narrower linear range as compared to the Abbott assay, and needs to be improved.

Applications

This comparative study will be helpful for domestic manufacturers in developing countries to improve the quality of their products.

Peer review

The authors compared the sensitivity and linear range of two HBV DNA quantification assays, the Abbott assay presented a relative higher sensitivity and a wider linear range in clinical samples. The conclusions will be beneficial for physicians to select the appropriate assay to quantify HBV DNA levels in chronic HBV- infected patients, especially in developing countries.

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ORIGINAL ARTICLE

Connexin 32 and *43* promoter methylation in *Helicobacter pylori*-associated gastric tumorigenesis

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Author contributions: Wang Y and Huang LH contributed equally to this work and should be considered as co-first authors; Wang Y detected the expression and methylation of *Connexin 32* and *43* and wrote the manuscript; Huang LH guided the detection of *Connexin 32* and *43* expression and methylation, and translated and revised the manuscript; Xu CX designed the study, performed the endoscopic procedures, collected specimens and guided the writing and revising of the manuscript; Xiao J, Zhou L, Cao D, and Liu XM collected the specimens; Qi Y performed the *H. pylori* culture.
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Abstract

AIM: To explore the mechanism of abnormal *Connexin* (*Cx*) *32* and *Cx43* expression in the gastric mucosa after *Helicobacter pylori* (*H. pylori*) infection.

METHODS: Biopsy specimens of gastric mucosa in different gastric carcinogenesis stages with *H. pylori* infection, that is, non-atrophic gastritis (NAG; n = 24), chronic atrophic gastritis (CAG; n = 25), intestinal metaplasia (IM; n = 28), dysplasia (DYS; n = 24), and gastric cancer (GC; n = 30), as well as specimens of normal gastric mucosa without *H. pylori* infection (NGM; n = 25), were confirmed by endoscopy and pathological examination. *Cx32* and *Cx43* mRNA expression was detected by real-time polymerase chain reaction (PCR). *Cx32* and *Cx43* promoter CpG island methylation status was determined by methylation-specific PCR (MSP), bisulfite PCR sequencing (BSP) and MassArray methods.

RESULTS: The relative mRNA expression levels in the gastric mucosa of patients with NGM, NAG, CAG, IM, DYS and GC were 0.146 ± 0.011, 0.133 ± 0.026, 0.107 ± 0.035, 0.039 ± 0.032, 0.037 ± 0.01 and 0.03 ± 0.011 for *Cx32*; and 0.667 ± 0.057, 0.644 ± 0.051, 0.624 ± 0.049, 0.555 ± 0.067, 0.536 ± 0.058 and 0.245 ± 0.121 for *Cx43*, respectively, which were gradually decreasing and significantly different (GC *vs* NGM: $P < 0.001$ for *Cx32*, $P < 0.001$ for *Cx43*). The promoter methylation levels in the gastric mucosa from NGM to GC stages by MSP were 38.8% ± 9.0%, 43.1% ± 9.4%, 56.5% ± 3.1%, 64.4% ± 9.7%, 72.5% ± 4.2% and 79.6% ± 6.8% for *Cx32*; and 49.0% ± 3.9%, 58.1% ± 5.0%, 66.5% ± 7.9%, 74.0% ± 8.8%, 78.3% ± 3.6% and 88.7% ± 6.2% for *Cx43*, respectively, which were gradually increasing and significantly different ($P = 0.039$, $P = 0.019$). The promoter methylation levels by BSP and MassArray exhibited similar trends. *Cx32* and *Cx43* mRNA expression was negatively correlated with promoter methylation status and gastric carcinogenesis stages ($P < 0.001$, $P = 0.016$).

CONCLUSION: *Cx32* and *Cx43* mRNA expression decreased gradually during *H. pylori* infection-associated gastric carcinogenesis, and it is associated with hypermethylation of these genes' promoter.

Key words: Gastric cancer; *Helicobacter pylori*; *Cx32*; *Cx43*; DNA methylation

Core tip: The relationship between *Connexin* (*Cx*) *32*

and *Cx43* mRNA expression and gene promoter methylation at different gastric carcinogenesis stages with *H. pylori* infection, that is, non-atrophic gastritis, atrophic gastritis, intestinal metaplasia, dysplasia, and gastric cancer, is not clear. Here, gastric mucosa biopsy specimens from these carcinogenic stages, as well as normal gastric mucosa without *H. pylori* infection, were examined for *Cx32* and *Cx43* mRNA expression and promoter methylation by real-time polymerase chain reaction and methylation detection. *Cx32* and *Cx43* mRNA expression decreased gradually during gastric carcinogenesis, and it is associated with hypermethylation of these genes' promoter.

Wang Y, Huang LH, Xu CX, Xiao J, Zhou L, Cao D, Liu XM, Qi Y. *Connexin 32* and *43* promoter methylation in *Helicobacter pylori*-associated gastric tumorigenesis. *World J Gastroenterol* 2014; 20(33): 11770-11779 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i33/11770.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i33.11770

INTRODUCTION

Helicobacter pylori (*H. pylori*) infection is an important risk factor for gastric cancer (GC)[1], with its carcinogenic mechanisms not yet fully understood[2,3]. *Connexin* (*Cx*) *32* and *Cx43* are key members of gap junctions between gastric epithelial cells, showing a gradual down-regulation trend from normal mucosa to precancerous lesions and GC[4]. We have found that the decrease in *Cx32* and *Cx43* expression in precancerous lesions and GC is associated with *H. pylori* infection[5], coculture of gastric epithelial cells with *H. pylori* reduces expression of *Cx43*[6], and eradication of *H. pylori* upregulates *Cx32* and *Cx43* expression in precancerous lesions[7]. However, the mechanisms by which *H. pylori* infection decreases *Cx32* and *Cx43* expression are unclear. Inactivation of gastric tumor suppressor genes, such as *CDX2*, *RASSF1A* and *P16*(*INK4A*), is induced by promoter hypermethylation[8-10]. In this study, we observed *Cx32* and *Cx43* mRNA expression and its relationship with the promoter methylation status in different stages of GC, and from the *Cx32* and *Cx43* gene methylation perspective, to explore the mechanism of abnormal *Cx32* and *Cx43* expression after *H. pylori* infection and its role in the occurrence and development of GC.

MATERIALS AND METHODS

Patients and tissues

A total of 1550 patients underwent endoscopic and pathological examinations because of upper gastrointestinal symptoms between September 2011 and April 2012 in the Third Xiangya Hospital, Central South University, Changsha, China. Fifty cases at each stage of gastric carcinogenesis with *H. pylori* infection, *i.e.* non-atrophic gastritis (NAG), chronic atrophic gastritis (CAG), intestinal metaplasia (IM), atypical hyperplasia (dysplasia, DYS) and GC, were screened; and 50 cases of normal gastric mucosa from age-matched subjects without *H. pylori* infection (NGM) in the same period were chosen as controls. Patients with gastric surgery or those taking antibiotics, nonsteroidal anti-inflammatory drugs, proton-pump inhibitors (PPIs), or histamine receptor (H_2) antagonists within 1 mo before endoscopy were excluded. Signed informed consent was obtained from all patients and controls, and the study was approved by the hospital medical ethics committee. Endoscopic and pathological diagnosis was made according to the 8th edition of the Cecil Essentials of Medicine[11], Chinese consensus on chronic gastritis[12] and Chinese guidelines for diagnosis and treatment of gastric cancer (2011 edition)[13]. Generally, the endoscopic findings of NAG included mucosal congestion and edema, accompanied with little hemorrhage and erosion, and those of CAG included a thinning mucous layer, shallowing or disappearing folds, visibility of the submucosal vascularity, and fine granules on the surface. The pathological findings of NAG included necrosis of the superficial mucosal epithelium and infiltration of lymphocytes and plasma cells in the lamina propria, and those of CAG included shrinking gastric glands with a reduced number and shallowing of gastric pits. IM was identified by replacement of gastric epithelium by intestinal epithelium, accompanied with goblet cells secreting acidic mucus, absorptive epithelial cells with striated edge, and Paneth cells. DYS was identified by proliferation of atypical cells, but it was not sufficient to be diagnosed as cancer. GC was identified as cancerous tissues infiltrating the mucosal, submucosal or entire layers, taking on polypoid, ulcerous, and diffuse infiltrative types.

Any two positives of rapid urease test, ^{14}C-urea breath test and histological examination, or positive *H. pylori* by culture were identified as *H. pylori* infection, and if these four tests were all negative, the patient was identified as being without *H. pylori* infection. Four pieces of lesioned or normal gastric mucosa biopsies were taken by gastroscopy, and mRNA and DNA were extracted for *Cx32* and *Cx43* mRNA expression and methylation detection. According to the quantity of mRNA and DNA, as well as no significant difference in sex, age and disease duration, there were 25 cases of NGM, 24 of NAG, 25 of CAG, 28 of IM, 24 of DYS, and 30 of GC, which were screened for *Cx32* and *Cx43* expression and methylation. Table 1 shows the clinical characteristics of the study population.

Reagents

Total RNA extraction and reverse transcription reagents were purchased from Toyobo (Osaka, Japan); Wizard Genomic DNA purification kit was purchased from Promega (Madison, WI, United States); the EpiTect Bisulfite Kit was purchased from Qiagen (Germany); and methylase (M.*Sss*I) was purchased from New England Biotech (United States).

Table 1 Sex, age and disease duration of cases of each stage (mean ± SD)

Stage	*n*	M	F	Age, yr	Disease duration (yr)
NGM without *H. pylori* infection	25	14	11	54.12 ± 8.21 (45-60)	0.85 ± 0.64 (0.12-1.23)
NAG with *H. pylori* infection	24	13	11	56.44 ± 11.29 (47-65)	0.85 ± 0.35 (0.37-1.03)
CAG with *H. pylori* infection	25	11	14	55.90 ± 7.80 (45-66)	0.73 ± 0.24 (0.13-0.90)
IM with *H. pylori* infection	28	12	16	52.16 ± 8.59 (48-68)	0.90 ± 0.65 (0.47-1.04)
DYS with *H. pylori* infection	24	12	12	54.05 ± 7.36 (48-69)	0.73 ± 0.57 (0.56-1.15)
GC with *H. pylori* infection	30	17	13	55.43 ± 10.33 (46-73)	0.60 ± 0.46 (0.10-1.04)

NGM: Normal gastric mucosa; NAG: Non-atrophic gastritis; CAG: Chronic atrophic gastritis; IM: Intestinal metaplasia; DYS: Dysplasia; GC: Gastric cancer.

Table 2 *Cx32* and *Cx43* primer sequences, amplified fragment size and annealing temperature

Method	Gene			Primer sequence (5′→3′)	Amplified fragment size (bp)	Annealing temperature (℃)
Real-time RT-PCR	*Cx32*		F:	ATGAACTGGACAGGTTTGTAC	302	56
			R:	ATGTGTTGCTGGTGCAGCCA		
	Cx43		F:	TGCAGCAGTCTGCCTTTCGTTG	219	56
			R:	CCATCAGTTTGGGCAACCTTG		
	β-actin		F:	TGGACTTCGCAGCACAGCAGATGG	289	56
			R:	ATCTCCTTCTGCATCCTGTCG		
MSP	*Cx32*	M	F:	GGGGCGGGTGCGGCGAT	245	64
			R:	CTCCGCGCCTACGTCCC		
		U	F:	GGGGTGGGTGTGGTGAT	245	64
			R:	CTCCACACCTACATCCCAA		
	Cx43	M	F:	AAATTGTAATATTTGGGTTTCAGCGC	156	58
			R:	AATAACGCCATCTCTACTCACCG		
		U	F:	TTTTAAAATTGTAATATTTGGGTTTCAGTGT	161	56
			R:	AATAACACCATCTCTACTCACCACA		
BSP	*Cx32*		F:	GGTTATTTTTTTGGTGGGGTTATG	313	58
			R:	ACCCAAACAAATCCCCTATAATCTC		
	Cx43		F:	TGTTTTTTAAAATTGTAATATTTGGGTTTA	377	56
			R:	AAAAACAAACTCATCTAACCTTCCTATTC		
MassArray	*Cx32*		F:	CAGTTT CAGCAGTTTTTGGGTTTTTTGG	484	60
			R:	TAACTCCCTATCCCCTAACTCCTTA		
	Cx43		F:	ATGTTTTTGCAGGTTGGATCAGGAAAT	447	60
			R:	ACCAACAAATAAAAACAAAATTATTCC		

Primers

The primers for detection of *Cx32* and *Cx43* expression and promoter methylation were designed with Primer5 [for real-time reverse transcription (RT)-polymerase chain reaction (PCR), including internal reference β-actin], MethPrimer [for methylation-specific PCR (MSP) and bisulfite sequencing PCR (BSP)], and EpiDesigner (for MassArray). The primers were synthesized by Shanghai Sangon Biotech (China) (Figure 1, Table 2).

Detection of Cx32 and Cx43 mRNA expression by real-time RT-PCR

The biopsy tissues of gastric mucosa were ground in liquid nitrogen, TRIzol was added to extract total RNA, and 1 μg total RNA was reversely transcribed to cDNA. With 2 μL cDNA as a template, real-time RT-PCR was carried out using SYBR qPCR Mix under the following conditions: 94 ℃ 4 min; 94 ℃ for 30 s, 56 ℃ for 45 s, 72 ℃ for 45 s, for 45 cycles; and 72 ℃ for 5 min. β-Actin was used as an internal reference, and nuclease-free water as a negative control. The relative mRNA expression was calculated according to the $2^{-\Delta Ct}$ formula.

Detection of Cx32 and Cx43 promoter methylation

DNA was extracted from the tissue with Wizard DNA purification kit, and then bisulfite-modified according to the steps in EpiTect Bisulfite Kit. DNA of normal human peripheral blood lymphocytes was methylated by M.*Sss*I methylation enzyme (thus all the GC-sites were methylated), bisulfite-modified and acted as an all-site methylation positive control. The following three methods were used to detect *Cx32* and *Cx43* promoter methylation.

MSP method: After the tissue DNA was bisulfite modified, PCR was carried out using MSP primers under the following conditions: 95 ℃ for 10 min; 94 ℃ for 15 s, annealing temperature for 30 s, 72 ℃ for 30 s, for 38 cycles; and 72 ℃ for 10 min. The PCR products were electrophoresed on 2% agarose gel and imaged. From the gray values of methylation and unmethylation bands, the methylation level was calculated by the formula [M/(M + U) × 100%].

BSP sequencing method: The PCR reaction mixture

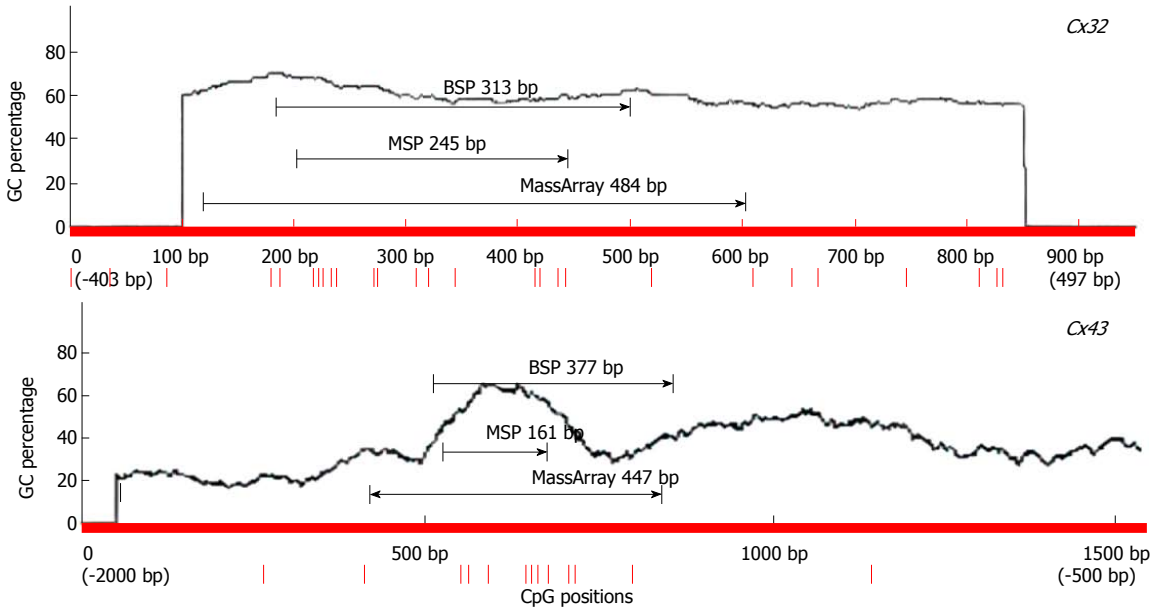

Figure 1 Location of primers for polymerase chain reaction for methylation detection.

(25 μL) contained 2 μL bisulfite-modified DNA template, 12.5 μL TaKaRa Premix Taq HS, 1 μL each 10 μmol/L forward and reverse primers, and 8.5 μL deionized distilled water. The PCR products were identified by electrophoresis, and sent to Huada Biotechnology (Shenzhen, China) for sequencing. The peak height ratio of the sulfonated methyl-CpG site still as C to the sum of C and the sulfonated non-methylated CpG site changed as T was calculated as the degree of methylation [*i.e.*, C/(C+T) × 100%].

MassArray method: Genomic DNA after bisulfite modification was amplified with MassArray primers. The PCR products were introduced with T7 promoter sequence in the Beijing Bio-Miao Biotech Company, then *in vitro* transcribed to RNA products, processed by T-base-specific cleavage, and small RNA fragments were obtained. Flight mass spectrometry (MALDI-TOF) was used to detect the molecular weight of each fragment, and the methylation data were outputted with EpiTyper software.

Statistical analysis

All data were processed with SPSS 16.0 software and shown as mean ± SD. Averages of multiple samples were compared using univariate analysis of variance, and correlation analysis of ranked data was tested by the Spearman rank correlation method. $P < 0.05$ was considered statistically significant.

RESULTS

Cx32 and Cx43 mRNA expression profiles at different gastric carcinogenesis stages with H. pylori infection

For cases with endoscopic and pathological confirmation and high mRNA quality (25 NGM, 24 NAG, 25 CAG, 28 IM, 24 DYS and 30 GC), the relative mRNA expression in the gastric mucosa was 0.146 ± 0.011, 0.133 ± 0.026, 0.107 ± 0.035, 0.039 ± 0.032, 0.037 ± 0.01 and 0.03 ± 0.011 for *Cx32*; and 0.667 ± 0.057, 0.644 ± 0.051, 0.624 ± 0.049, 0.555 ± 0.067, 0.536 ± 0.058 and 0.245 ± 0.121 for *Cx43* (Figure 2). *Cx32* and *Cx43* mRNA expression decreased from NAG to GC stages with *H. pylori* infection ($P < 0.001$), and that at CAG, IM, DYS and GC stages was lower than that at NGM ($P < 0.008$; the largest in these comparisons), and that at IM, DYS and GC stages was lower than that at NAG and CAG stages ($P < 0.036$). Specially, *Cx43* mRNA expression at GC stage was lower than that at IM and DYS stages ($P < 0.001$).

Cx32 and Cx43 promoter methylation at different gastric carcinogenesis stages with H. pylori infection

MSP: Eight DNA samples of good quality were selected from 24 cases in each group for MSP detection, and the actual number of samples whose methylated and unmethylated bands were significant was six for NGM, six for NAG, seven for CAG, six for IM, seven for DYS and eight for GC. Their promoter methylation levels were 38.8% ± 9.0%, 43.1% ± 9.4%, 56.5% ± 3.1%, 64.4% ± 9.7%, 72.5% ± 4.2% and 79.6% ± 6.8% for *Cx32*; and 49.0% ± 3.9%, 58.1% ± 5.0%, 66.5% ± 7.9%, 74.0% ± 8.8%, 78.3% ± 3.6% and 88.7% ± 6.2% for *Cx43* (Figure 3). *Cx32* and *Cx43* promoter methylation levels gradually increased from NAG to GC stages with *H. pylori* infection ($P = 0.039$, $P = 0.019$), and those at CAG, IM, DYS and GC stages were higher than that at NGM ($P = 0.018$, $P = 0.013$), with the highest at GC stage. *Cx32* methylation level at GC stage was higher than those at NAG, CAG and IM stages ($P < 0.031$), and *Cx43* methylation level at GC stage was higher than those at NAG and CAG stages ($P < 0.027$).

BSP sequencing: The samples with good MSP bands

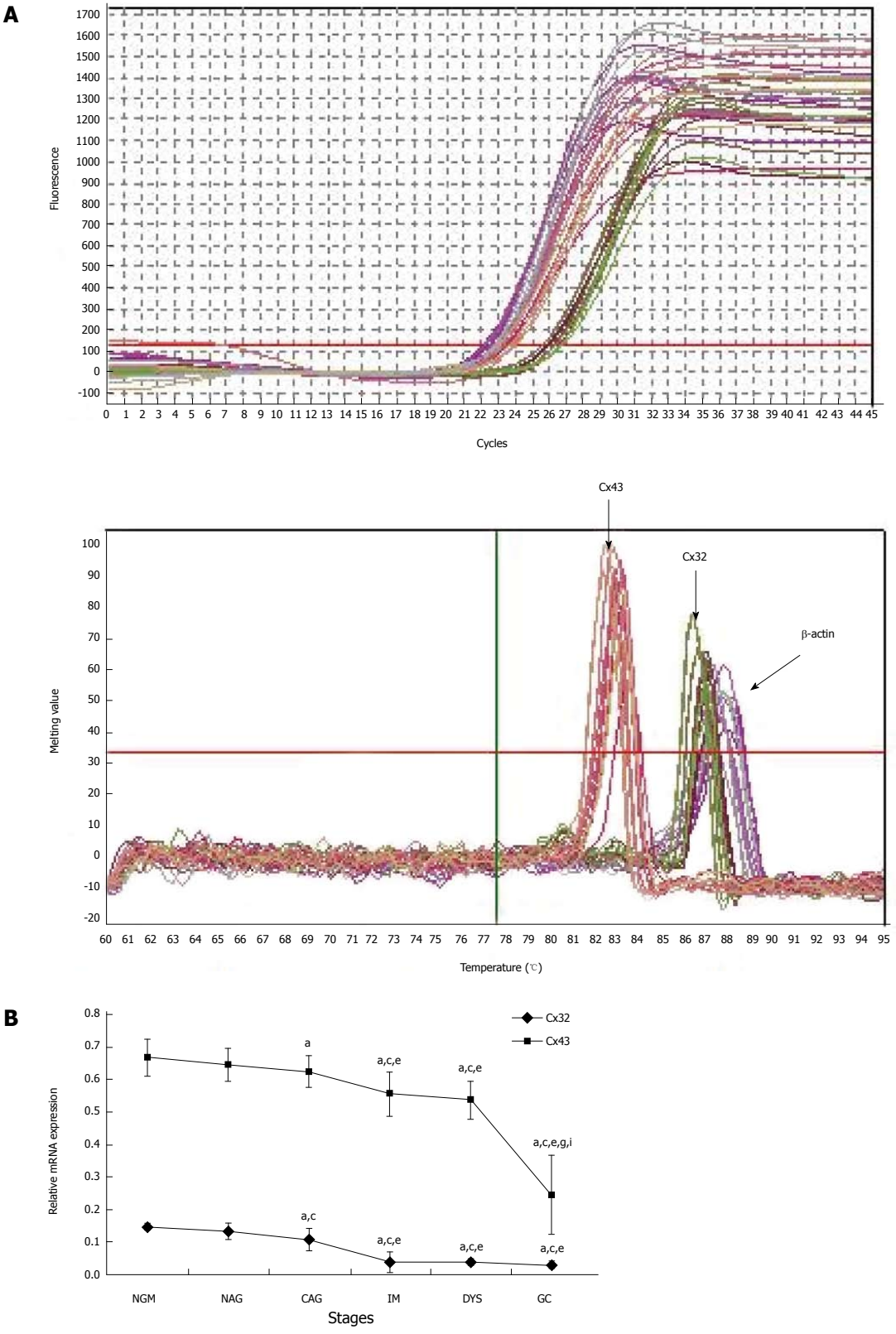

Figure 2 Real-time polymerase chain reaction results for *Cx32* and *Cx43* mRNAs. A: The amplification curve and melting curve of the real-time polymerase chain reaction; B: The relative expression of *Cx32* and *Cx43* mRNAs at different stages. $^{a}P < 0.05$ *vs* NGM; $^{c}P < 0.05$ *vs* NAG; $^{e}P < 0.05$ *vs* CAG; $^{g}P < 0.05$ *vs* IM; $^{i}P < 0.05$ *vs* DYS. NGM: Normal gastric mucosa; NAG: Non-atrophic gastritis; CAG: Chronic atrophic gastritis; IM: Intestinal metaplasia; DYS: Dysplasia; GC: Gastric cancer.

were selected for BSP reaction and sequencing. The BSP-amplified fragment from the *Cx32* gene CpG island had a length of 313 bp, containing 15 CpG sites, and the 5-15th CpG sites were detected by sequencing. The BSP amplification fragment from the *Cx43* gene CpG island had a length of 377 bp, containing 12 CpG sites, and the 5-12th CpG sites were detected by sequencing (Table 3). The peak height ratio of the sulfonated methyl-CpG site

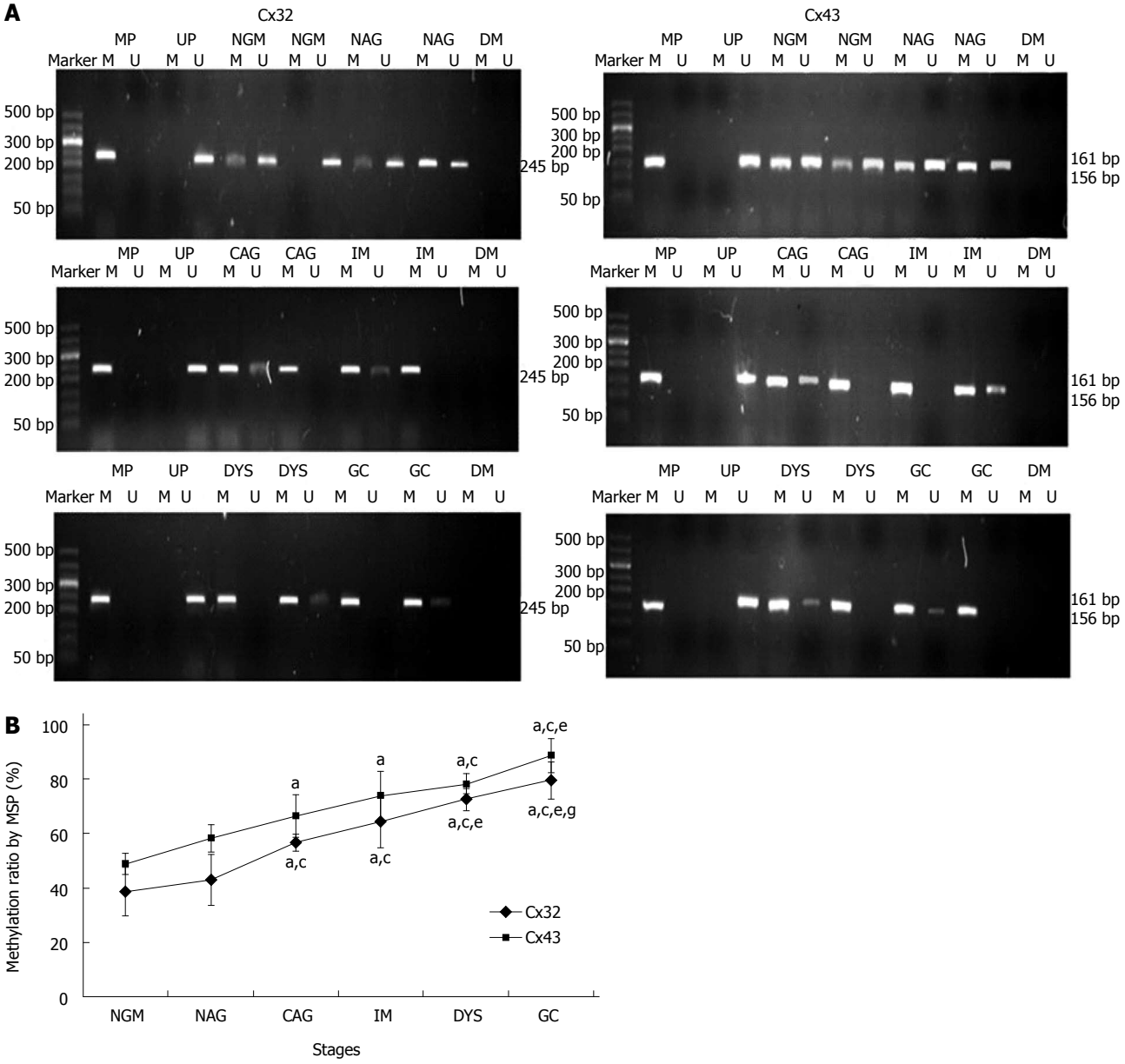

Figure 3 Methylation-specific polymerase chain reaction results for *Cx32* and *Cx43* promoters at different gastric carcinogenesis stages with *Helicobacter pylori* infection. A: The agarose gel electrophoresis of the methylation-specific polymerase chain reaction (MSP) bands. Marker: 50bp ladder; M: Methylated; U: Unmethylated; MP: Methylation positive control; UP: Unmethylated positive control; DW: Negative control. B: The methylation levels of *Cx32* and *Cx43* promoters at different stages by MSP method. [a]$P < 0.05$ *vs* NGM; [c]$P < 0.05$ *vs* NAG; [e]$P < 0.05$ *vs* CAG; [g]$P < 0.05$ *vs* IM; [i]$P < 0.05$ *vs* DYS. NGM: Normal gastric mucosa; NAG: Non-atrophic gastritis; CAG: Chronic atrophic gastritis; IM: Intestinal metaplasia; DYS: Dysplasia; GC: Gastric cancer.

still as C to the sum of this C and the sulfonated non-methylated CpG site changed as T was calculated as the degree of methylation. *Cx32* and *Cx43* methylation levels showed an increasing trend from NAG to GC stages with *H. pylori* infection ($P = 0.031$, $P = 0.040$), and those at CAG, IM, DYS and GC stages were higher than that at NGM ($P < 0.029$, $P < 0.03$), with the highest at GC. *Cx32* methylation level at GC stage was higher than those at NAG and CAG stages ($P < 0.018$), and *Cx43* methylation level at GC stage was higher than that at NAG stage ($P = 0.032$) (Figure 4, Table 4).

MassArray detection: The methylation of the CpG island was validated by MassArray method using one sample from each group. As shown in Figure 5, the amplified fragment with MassArray *Cx32* primers contained 18 CpG sites, the first, second and 15th CpG sites were not detected by the MassArray method, and the 3-4-5, 6-7, 8-9, 13-14, and 16-17 loci were in close proximity and only measured on average, so a total of nine data were obtained. For the 12 CpG sites in the *Cx43* amplified fragment, the second site was not detected, and the 6-7 and 10-11-12 loci were in close proximity and only measured on average, so a total of eight data were obtained. The average of the methylation levels of these loci is shown in Table 5. The average of the methylation levels at all *Cx32* loci showed an increasing trend from NAG to GC stages with *H. pylori* infection ($P = 0.037$), and that at IM, DYS

Table 3 *Cx32* and *Cx43* CpG loci detected by bisulfite polymerase chain reaction sequencing

Cx32	CpG	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	bp	28	34	37	45	49	83	86	120	132	155	227	231	247	253	255
		○	○	○	○	●	●	●	●	●	●	●	●	●	●	●
Cx43	CpG	1	2	3	4	5	6	7	8	9	10	11	12			
	bp	32	34	37	44	71	127	134	143	159	190	198	280			
		○	○	○	○	●	●	●	●	●	●	●	●			

"●" denotes the detected CpG site; "○"denotes the undetected CpG site.

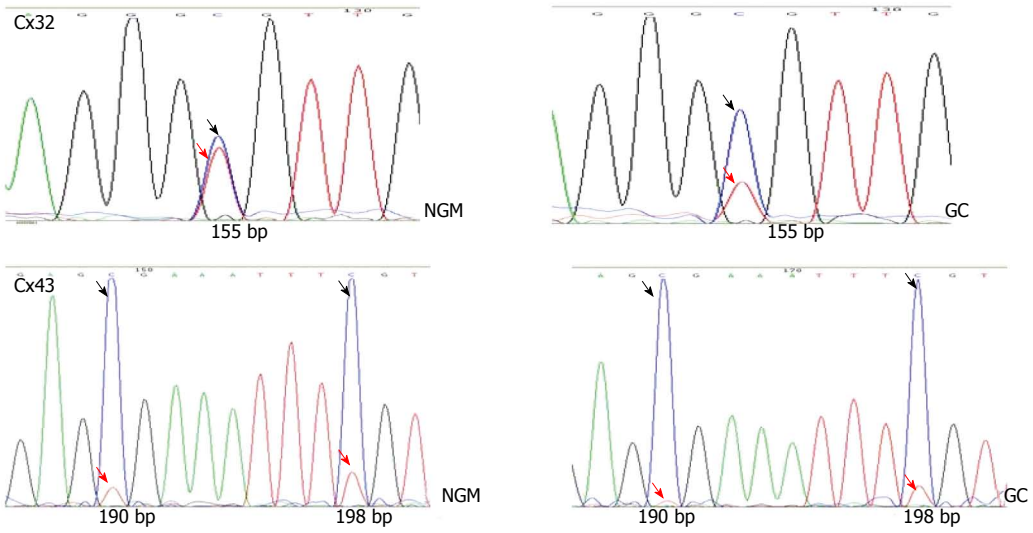

Figure 4 Screenshots of bisulfite polymerase chain reaction sequencing of the *Cx32* and *Cx43* promoter CpG islands. The upper two show the 10th *Cx32* CpG site (155bp), with the C/(C+T) ratio of 60% in NM and 80% at GC stage. The lower two shows the 10th and 11th *Cx43* CpG sites (190bp, 198bp). Dark arrow indicates the peak of the sulfonated methyl-CpG site still as C; red arrow indicates the sulfonated non-methylated CpG site changed as T.

Table 4 *Cx32* and *Cx43* promoter methylation status at different gastric carcinogenesis stages with *Helicobacter pylori* infection by bisulfite polymerase chain reaction sequencing method

	n	Cx32 (%)	Cx43 (%)
NGM without *H. pylori* infection	7	60.1 ± 5.9	75.5 ± 4.3
NAG with *H. pylori* infection	6	67.3 ± 4.9	82.9 ± 6.3
CAG with *H. pylori* infection	7	74.5 ± 7.5[a]	87.1 ± 5.4[a]
IM with *H. pylori* infection	7	84.2 ± 6.8[ace]	90.5 ± 9.3[ac]
DYS with *H. pylori* infection	6	82.3 ± 6.0[ac]	91.6 ± 8.3[ac]
GC with *H. pylori* infection	9	85.3 ± 9.7[ace]	92.0 ± 7.1[ac]

[a]$P < 0.05$ *vs* NM; [c]$P < 0.05$ *vs* NAG; [e]$P < 0.05$ *vs* CAG. NGM: Normal gastric mucosa; NAG: Non-atrophic gastritis; CAG: Chronic atrophic gastritis; IM: Intestinal metaplasia; DYS: Dysplasia; GC: Gastric cancer.

and GC stages was higher than that at NGM ($P < 0.028$); the methylation level at the 10-12th loci had an increasing trend from NAG to GC stages, while the methylation level of the remaining CpG sites did not change significantly. The average methylation levels at all *Cx43* loci showed an increasing trend from NAG to GC stages with *H. pylori* infection ($P = 0.045$), and those at DYS and GC stages were higher than that at NGM ($P < 0.041$). The methylation levels at the 3-5th loci tended to increase from NAG to GC stages, while the methylation levels of the remaining CpG sites did not change significantly.

Relationship between GC stages and Cx32 and Cx43 expression and methylation levels

Spearman rank correlation analysis showed that *Cx32* and *Cx43* mRNA expression at different stages of gastric carcinogenesis with *H. pylori* infection was negatively correlated with the methylation level of their promoters ($r = -0.653$, $P < 0.001$; $r = -0.367$, $P = 0.016$, respectively), and negatively correlated with gastric carcinogenesis stage ($r = -0.796$, -0.852, respectively, $P < 0.001$ for both).

DISCUSSION

Many studies have shown that expression of *Cx32* and *Cx43* shows a gradual downward trend from normal gastric mucosa to precancerous lesions and GC. *Cx32* and *Cx43* expression progresses from a high to a low level or no expression in the development of GC, and *Cx32* and *Cx43* abnormalities are an important molecular mechanism in the inhibition of gastric gap junction intercellular communication (GJIC)[4,14-16].

The relationship between *Cx32* and *Cx43* expression and *H. pylori* infection is less reported. The results of our previous clinical studies[5-7] have suggested that eradication of *H. pylori* infection may improve *Cx32* and *Cx43* expression, promote recovery of cell GJIC function, and delay or prevent development of precancerous lesions.

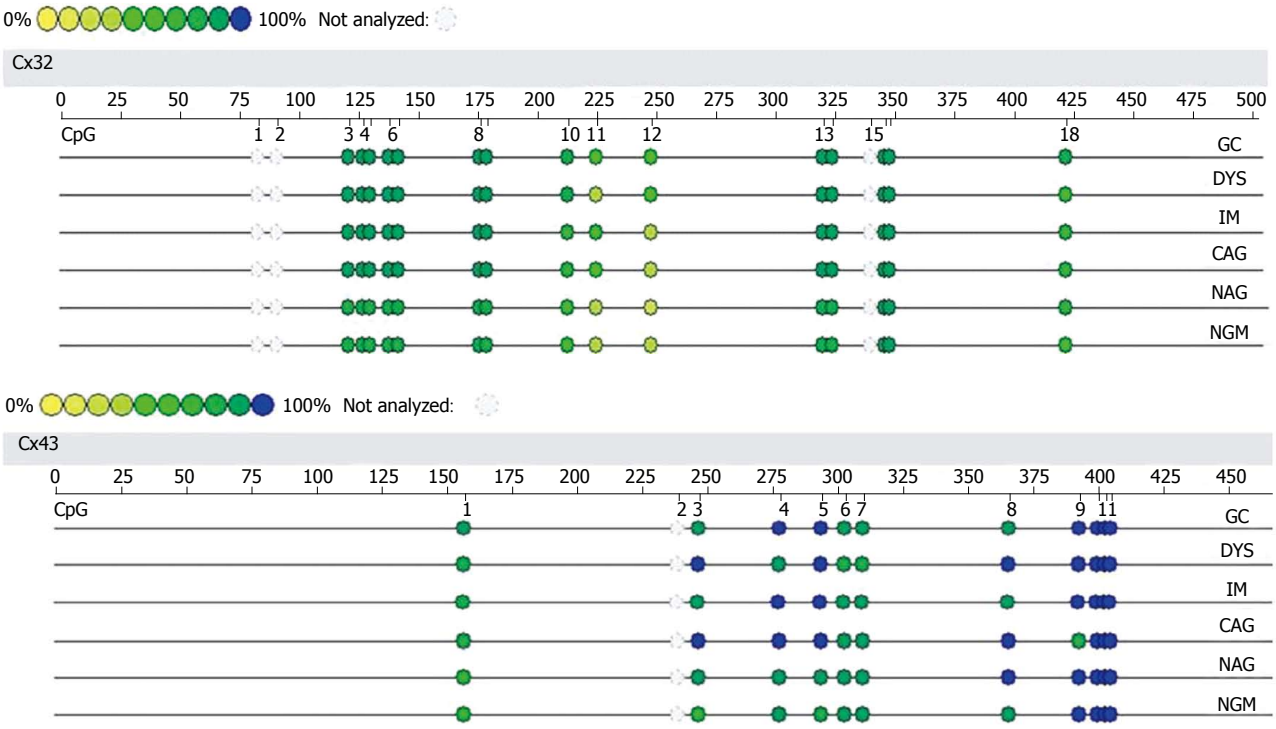

Figure 5 Methylation levels of *Cx32* and *Cx43* promoters at different gastric carcinogenesis stages with *Helicobacter pylori* infection by MassArray method. The validated length for *Cx32* gene was 484 bp, containing a total of 18 CpG sites (15 detected); the validated length for *Cx43* gene was 447 bp, containing a total of 12 CpG sites (11 detected).

Table 5 Methylation levels (%) of *Cx32* and *Cx43* CpG islands at gastric different stages with *Helicobacter pylori* infection detected by MassArray method

	Cx32 (9 loci)	Cx43 (8 loci)
NGM without *H. pylori* infection	55.0 ± 14.9	77.9 ± 16.8
NAG with *H. pylori* infection	61.2 ± 17.9	86.0 ± 12.5
CAG with *H. pylori* infection	69.9 ± 16.2	88.6 ± 9.9
IM with *H. pylori* infection	72.4 ± 19.2[a]	89.1 ± 9.6
DYS with *H. pylori* infection	72.6 ± 19.2[a]	90.0 ± 9.3[a]
GC with *H. pylori* infection	76.0 ± 20.1[a]	91.6 ± 8.2[a]

[a]$P < 0.05$ *vs* NGM. NGM: Normal gastric mucosa; NAG: Non-atrophic gastritis; CAG: Chronic atrophic gastritis; IM: Intestinal metaplasia; DYS: Dysplasia; GC: Gastric cancer.

However, the pattern and mechanism by which *H. pylori* infection causes the change of *Cx32* and *Cx43* expression in gastric epithelial cells in the development of the inflammation-carcinoma chain are unclear.

In this study, we found that gastric *Cx32* and *Cx43* mRNA expression was downregulated from the initial CAG stage of *H. pylori* infection to latter carcinogenesis stages, which may have caused the decline in GJIC function, leading to the development of GC. *H. pylori* is deemed to be the first category of carcinogen for gastric cancer[17], and current studies suggest that eradication therapy of *H. pylori* should be carried out at early stages of GC. Treatment before the occurrence of precancerous lesions can reduce the risk of GC, and treatment at the precancerous stage significantly decreases its role in prevention of GC[18-20]. From the profiles of *Cx32* and *Cx43* mRNA expression, we provide a rationale that *H. pylori* eradication therapy should be carried out before the occurrence of CAG, that is, before the decline in *Cx32* and *Cx43* expression and GJIC function (NAG stage), thus the effect of preventing the occurrence and development of precancerous lesions and GC may be improved.

H. pylori infection can cause promoter CpG island hypermethylation of a variety of genes, such as *CDH1*, *p14*, *p16*, *APC* and *COX2*, and the methylation can be reversed after *H. pylori* eradication[21,22], suggesting that gene hypermethylation is associated with *H. pylori* infection[23-25], or *H. pylori* infection may be an inducer for some gene hypermethylation[26,27]. However, it has not been reported whether *H. pylori* infection causes *Cx32* and *Cx43* methylation.

Our research showed that *Cx32* and *Cx43* promoter methylation exhibited an increasing trend from NAG to GC stages with *H. pylori* infection, with the highest at the GC stage. Data from the MSP method were more significant than from those BSP or MassArray methods, mainly because MSP used direct PCR of several CpG sites in the primer regions, while different fluorescence or mass spectrum quantification and many CpG sites were considered in BSP sequencing or MassArray method. *Cx32* and *Cx43* mRNA expression was negatively correlated with methylation and gastric carcinogenesis stage, suggesting that *Cx32* and *Cx43* promoter hypermethylation may be an important mechanism for the reduction of *Cx32* and *Cx43* expression and occurrence of GC. Hypermethylated promoter binds specific chromosome

remodeling proteins and suppresses the transcription of genes[8,28-30].

Currently, many molecular technologies are being developed and applied for cancer epigenetics[31]. In GC patients, *TGF-β1* promoter is methylated[32], and in colorectal cancer, underexpression of *LATS1* is associated with promoter hypermethylation[33]. Intervention with demethylation drugs has been reported to reactivate the expression of gastric tumor suppressor genes such as *CDX2*, *RASSF1A* and *P16* (*INK4A*), and restore their functions[8-10]. *H. pylori* infection may cause *Cx32* and *Cx43* promoter hypermethylation to decrease their expression, then inhibit the GJIC function, and induce GC. Promoter methylation can be reversed, thus, it is expected that we can treat against promoter methylation to restore GJIC function, providing new therapies for *H. pylori* infection-related GC. Based on our research, the treatment of GC may include eradiation of *H. pylori*, adding DNA-demethylation agents (*e.g.*, 5-azacytidine), and overexpression of *Cx32* and Cx*43* to compensate the decrease of *Cx32* and Cx*43* in the carcinogenesis.

In summary, *Cx32* and *Cx43* expression at the CAG stage of *H. pylori* infection began to decrease, suggesting that *H. pylori* eradication therapy before the CAG stage could effectively prevent the occurrence of precancerous lesions and GC. *Cx32* and *Cx43* promoter hypermethylation may be an important mechanism of the decrease of *Cx32* and *Cx43* expression after *H. pylori* infection, and provides a new target for the demethylation treatment of GC.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) infection is an important risk factor for gastric cancer (GC), yet its carcinogenic mechanism is not yet fully understood. Connexin (Cx) 32 and Cx 43 are key members of gap junctions between gastric epithelial cells. The authors have found that the decrease of *Cx32* and *Cx43* expression in precancerous lesions and GC is associated with *H. pylori* infection, but the mechanisms by which *H. pylori* infection leads to the decrease of *Cx32* and *Cx43* expression are unclear.

Research frontiers

It is important to understand the epigenetic mechanism of occurrence of GC. Many studies have demonstrated that the expression of *Cx32* and *Cx43* shows a gradual downward trend from normal gastric mucosa to precancerous lesions and GC. *Cx32* and *Cx43* abnormalities are the important molecular mechanism of the inhibition of gastric gap junction intercellular communication (GJIC) which may then lead to gastric carcinogenesis. *H. pylori* infection can cause promoter CpG island hypermethylation of a variety of genes, such as *CDH1*, *p14*, *p16*, *APC* and *COX2*, and methylation can be reversed after *H. pylori* eradication.

Innovations and breakthroughs

Following previous work showing that the decrease of *Cx32* and *Cx43* expression in precancerous lesions and GC was associated with *H. pylori* infection, the present study found that *Cx32* and *Cx43* mRNA expression was negatively correlated with promoter methylation and gastric carcinogenesis stage. This suggests that *Cx32* and *Cx43* promoter hypermethylation may be an important mechanism in the reduction of *Cx32* and *Cx43* expression and occurrence of GC. This can help find new targets for applying appropriate means to control the incidence of GC.

Applications

This study shows that *Cx32* and *Cx43* promoter CpG islands are gradually methylated at gastric carcinogenesis stages with *H. pylori* infection. Promoter methylation can be reversed, therefore, it is expected that we can treat against promoter methylation to restore GJIC function, providing new therapies for *H. pylori* infection-related GC.

Terminology

CpG islands: CpG rich areas located in the promoter regions of many genes; CpG island methylation: the addition of a methyl group to a cytosine residue that lies next to guanine within CpG dinucleotides. *Cx32* and *43*: key members of gap junctions between gastric epithelial cells. Gastric carcinogenesis stages: graded according to the diagnoses by endoscopy and pathology, including non-atrophic gastritis, chronic atrophic gastritis, intestinal metaplasia, dysplasia, and GC.

Peer review

The hypothesis was sound, the experiments were well designed and the results supported the conclusions. This is a well written manuscript about new therapeutic targets for treatment of GC and its different carcinogenesis stages with *H. pylori* infection. The analyses were performed well and are clearly described in the manuscript.

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RESEARCH REPORT

Characterization of monocarboxylate transporter activity in hepatocellular carcinoma

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Abstract

AIM: To assess the immunoexpression of hypoxia-related markers in samples from cirrhosis and primary and metastatic hepatocellular carcinoma (HCC).

METHODS: From a total of 5836 autopsies performed at the Pathology Department - University of Sao Paulo School of Medicine Hospital - from 2003 to 2009, 188 presented primary liver tumors. Immunohistochemical reactivity for monocarboxylate transporters (MCTs)-1, 2 and 4, CD147 and glucose transporter-1 (GLUT1) was assessed in necropsies from 80 cases of HCC. Data were stored and analyzed using the IBM SPSS statistical software (version 19, IBM Company, Armonk, NY). All comparisons were examined for statistical significance using Pearson's χ^2 test and Fisher's exact test (when $n < 5$). The threshold for significant P values was established as $P < 0.05$.

RESULTS: Plasma membrane expression of MCT4 and overall expression of GLUT1 showed progressively higher expression from non-neoplastic to primary HCC and to metastases. In contrast, overall expression of MCT2 was progressively decreased from non-neoplastic to primary HCC and to metastases. MCT1 (overall and plasma membrane expression), MCT2 and CD147 plasma membrane expression were associated with absence of cirrhosis, while plasma membrane expression of CD147 was also associated with absence of HBV infection. MCT2 overall expression was associated with lower liver weight, absence of metastasis and absence of abdominal dissemination. Additionally, MCT4 plasma membrane positivity was strongly associated with Ki-67 expression.

CONCLUSION: MCT4 and GLUT1 appear to play a role in HCC progression, while MCT2 is lost during progression and associated with better prognosis.

Key words: Hepatocellular carcinoma; Monocarboxylate transporters; Glycolysis; Cirrhosis; Glucose transporter-1

Core tip: This paper describes, for the first time, the role of monocarboxylate transporters in hepatic carcinoma. The characterization of monocarboxylate transporter activity in acidic metabolism of primary and metastatic hepatocellular carcinoma microenvironment was studied in necropsy material and allowed us to precisely evaluate the impact of the more acidic microenvironment that is potentially maintained by monocar-

boxylate transporter 4 and glucose transporter-1 during hepatocellular carcinoma progression.

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INTRODUCTION

Oxygen is the essential final electron acceptor of energy metabolism. Under physiological conditions, the mean oxygen tension (dissolved free oxygen concentration) is 74-104 mmHg in arterial blood and 34-46 mmHg in venous blood. The "double vascular pattern" in the liver results in a physiological oxygen tension gradient, from 60-65 mmHg in peri-portal blood falling to approximately 30-35 mmHg in centrilobular regions, thus rendering liver parenchyma especially vulnerable to hypoxia[1]. Hypoxia inducible factor 1α (HIF-1 α) is the major regulator hypoxia modulation and targets many enzymes of the glycolytic pathway, including glucose transporter-1 (GLUT1), lactate dehydrogenase A (LDH-A) and monocarboxylate transporters, and especially monocarboxylate transporter (MCT)-4[2,3].

The microenvironment of solid tumors tends to become acidic due to the high rate of glycolysis maintained by cancer cells, thus resulting in the production of high amounts of lactate and leading to acidification of the extracellular milieu[3]. Warburg[4] was the first to suggest that cancer cells prioritize the glycolytic pathway for energy production, even in the presence of sufficient oxygen, a phenomenon that is now known as the "Warburg effect".

MCTs have been recognized to play a key role in the maintenance of this glycolytic metabolism by mediating lactate efflux from cancer cells. Overexpression of one or more MCT isoforms, especially MCT1 and MCT4, has been implicated in tumor prognosis, and MCTs have thus been suggested as potential therapeutic targets[3,5]. Our group has been studying the expression of these MCT isoforms as well as their chaperone, CD147, known to be essential for MCT activity and plasma membrane expression, in different types of human cancers[3]. We found upregulation of MCT1 and MCT4 in the plasma membrane of colorectal cancer[6], upregulation of MCT1, MCT4 and CD147 in cervical cancer[7,8], upregulation of MCT1 in breast cancer[9] and upregulation of MCT1 and CD147 in glioblastomas[5], when compared to the corresponding non-neoplastic tissues. In contrast, there was a downregulation of MCT4 in gastric cancer[10] and a downregulation of MCT1 and CD147 in prostate cancer[11]. We also found important associations between MCT overexpression and the clinicopathological data of the cases, mostly with aggressiveness parameters[3,12-14].

Considering the susceptibility of liver to hypoxia, the growing relevance of hepatocellular carcinoma (HCC) worldwide[15,16] and the potential impact of MCTs in the development of several solid tumors, we sought herein to investigate the expression of MCT1, 2 and 4, the MCT chaperone CD147 and the glycolytic marker GLUT1 in a necropsy series of 73 well-characterized cases of advanced HCC and corresponding non-neoplastic samples, searching for a possible role of glycolytic metabolism in the development of advanced hepatocellular carcinoma.

MATERIALS AND METHODS

Autopsy samples and data

From a total of 5836 autopsies performed at the Pathology Department - University of Sao Paulo School of Medicine Hospital - from 2003 to 2009, 188 presented primary liver tumors. Excluding 65 cholangiocarcinomas, one combined hepatocholangiocarcinoma, one epithelioid hemangioendothelioma and 13 other malignant neoplasms, 108 cases were diagnosed as HCC. A review of these cases was performed in accordance with the investigative protocols of the Institutional Review Boards of the University of Sao Paulo's School of Medicine as part of the doctoral thesis recently presented by one of the authors[17]. Sufficient viable tissue samples as well as clinical data (gender, age, viral hepatitis, alcoholism, previous treatment) from medical records and from autopsy reports were retrieved in 80 HCC cases. Detailed pathological data were recorded - liver weight, gross appearance, number/size of primary tumors or metastases and large portal or hepatic vein invasion - and complemented by slide review (histological grade and pattern). Paraffin tissue blocks from these 80 HCC cases were used for tissue microarray (TMA) construction and immunohistochemistry studies.

TMA construction

Primary HCC and extra-hepatic metastasis samples were selected upon microscopic review (ASFS) and 3 cores of each sample were spotted in two TMAs (1.0 mm cores). For heterogeneous tumors, different areas were separately cored. When more than one primary HCC was present in a single case, all tumors were sampled, but data were computed for the largest one. Fewer spots were used only when the size of the metastasis was limiting (< 0.5 cm). Available non-neoplastic liver samples were selected as far from the tumor border as possible, usually in a different paraffin block, and cored in one TMA.

Immunohistochemistry reactions

Primary antibodies: As depicted in Table 1, primary antibodies against MCT1, MCT2, MCT4, CD147, and GLUT1 were standardized in our laboratory, as previously published[6,8,12].

Briefly, deparaffinized and rehydrated sections were subjected to specific conditions of heat-induced antigen

Table 1 Immunohistochemistry protocols used to characterize the proteins expression

Code	Industry	Dilution	Epitope retrieval	Amplification system
MCT1 (sc-365501)	Santa Cruz Biotechnology, Santa Cruz, CA, United States	1:500	EDTA (1 mmol/L, pH = 8); 98 ℃; 20 min	Vectastain® Elite ABC reagent (Burlingame, CA, United States)
MCT2 (sc-14926)	Santa Cruz Biotechnology, Santa Cruz, CA, United States	1:200	Citrate buffer (10 mmol/L, pH = 6); 98 ℃; 20 min	Ultravision Detection System Anti-polyvalent, HRP, Lab Vision Corporation, Fremont, CA, United States)
MCT4 (sc-50329)	Santa Cruz Biotechnology, Santa Cruz, CA, United States	1:500	Citrate buffer (10 mmol/L, pH = 6); 98 ℃; 20 min	Ultravision Detection System Anti-polyvalent, HRP, Lab Vision Corporation, Fremont, CA, United States)
CD147 (sc-71038)	Santa Cruz Biotechnology, Santa Cruz, CA, United States	1:400	EDTA (1 mmol/L, pH = 8); 98 ℃; 20 min	Vectastain® Elite ABC reagent (Burlingame, CA, United States)
GLUT1 (ab15309)	Abcam, Cambridge, United Kingdom	1:500	Citrate buffer (10 mmol/L, pH = 6); 98 ℃; 20 min	Ultravision Detection System Anti-polyvalent, HRP, Lab Vision Corporation, Fremont, CA, United States)
Caspase 3 (3C SP03)	Diagnostic BioSystems (DBS) Pleasanton, CA, United States	1:160	Citrate buffer (10 mmol/L, pH = 6); 98 ℃; 40 min	NovoLink (Novocastra Laboratories Ltd, Newcastle Upon Tyne, United Kingdom)
Keratin 19 (K19, b170)	Novocastra Laboratories Ltd, Newcastle Upon Tyne, United Kingdom	1:300	Citrate buffer (10 mmol/L, pH = 6); 98 ℃; 40 min	NovoLink (Novocastra Laboratories Ltd, Newcastle Upon Tyne, United Kingdom)
Ki-67 (MIB-1)	(Dako, Glostrup, Denmark)	1:400	Citrate buffer (10 mmol/L, pH = 6); 98 ℃; 40 min	NovoLink (Novocastra Laboratories Ltd, Newcastle Upon Tyne, United Kingdom)
EGFR human (DAK-H1-WT)	(Dako, Glostrup, Denmark)	1:200	Citrate buffer (10 mmol/L, pH = 6); 98 ℃; 40 min	NovoLink (Novocastra Laboratories Ltd, Newcastle Upon Tyne, United Kingdom)

MCT: Monocarboxylate transporter; GLUT: Glucose transporter; EGFR: Epidermal growth factor receptor.

retrieval. After inactivation of endogenous peroxidases, tissue sections were incubated with protein blocking solution for 20 min and incubated with the primary antibody under the conditions specified in Table 1. Sections were then sequentially washed in PBS and incubated with biotinylated secondary antibody and enzyme-coupled reagent from R.T.U. Vectastain® Elite ABC (Burlingame, CA, United States) for MCT1 and CD147 or Ultravision Detection System Anti-polyvalent, HRP (Lab Vision Corporation, Fremont, CA) for MCT2, MCT4 and GLUT-1. All reactions were developed with 3,3'-diamino-benzidine (DAB+ Substrate System, DakoCytomation, Carpinteria, CA, United States) for 10 min. Negative controls were performed by using an appropriate serum control for the primary antibodies (n1698, DakoCytomation, Carpinteria, CA, United States) and colon carcinoma tissue was used as a positive control for MCT1, MCT2, MCT4 and CD147, and squamous cell laryngeal carcinoma for GLUT1. All tissue sections were counterstained with hematoxylin and permanently mounted. Immunoreactions for EGFR, Ki-67, Keratin 19 (putative markers of molecular classification of HCC recently proposed by Hoshida *et al*[18]) as well as for the apoptosis marker Caspase 3 were previously performed[17] and were herein compared to MCTs expression.

Immunohistochemical evaluation

Sections were semi-quantitatively scored for immunoreaction as follows: 0: 0% of immunoreactive cells; 1: < 5% of immunoreactive cells; 2: 5%-50% of immunoreactive cells; and 3: > 50% of immunoreactive cells. Additionally, staining intensity was scored semi-qualitatively as follows: 0: negative; 1: weak; 2: intermediate; and 3: strong. The final score was defined as the sum of both parameters (extent and intensity) and grouped as negative (score 0 and 2) and positive (score 3-6), as previously described[6]. Because being located in the plasma membrane is essential for the activity of these proteins, the presence of plasma membrane expression of MCTs, CD147 and GLUT1 was recorded. Immunohistochemical assessment was blindly performed by two independent observers (VAFA and ASFS) and discordant cases were discussed using a double-head microscope in order to determine the final score.

The semi-quantitation of each marker presented above was compared with the clinical-pathological variables and with the quantitative assessment of EGFR, Ki-67 and caspase 3 expression[17]. EGFR semi-quantitation assessed both the percentage of positive cells (0-100) and intensity of staining (1-3), thus leading to the score 0-300 and to the categories: negative < 10, positive ⩾ 10 and positive ⩾ 200 (hyper-expression), as recently published[19]. Ki67 values were assessed after counting 1000 cells; and caspase 3 expression was considered positive when more than 10 bodies were stained/10 HPF; or "loss of expression" (negative) when 10 or less bodies were stained/10 HPF. Keratin 19 expression was dichotomized in Positive or Negative reaction.

Statistical analysis

Data were stored and analyzed using the IBM SPSS statistical software (version 19, IBM Company, Armonk, NY). All comparisons were examined for statistical significance using Pearson's χ^2 test and Fisher's exact test (when $n < 5$). The threshold for significant P values was established as $P < 0.05$.

RESULTS

Figure 1 illustrates positive immunoreactions for the different markers analyzed in the different hepatic lesions. Data presented in Tables 2 and 3 show that the proteins were found in both the cytoplasm and the plasma membrane regions of cancer cells, at different frequencies. All

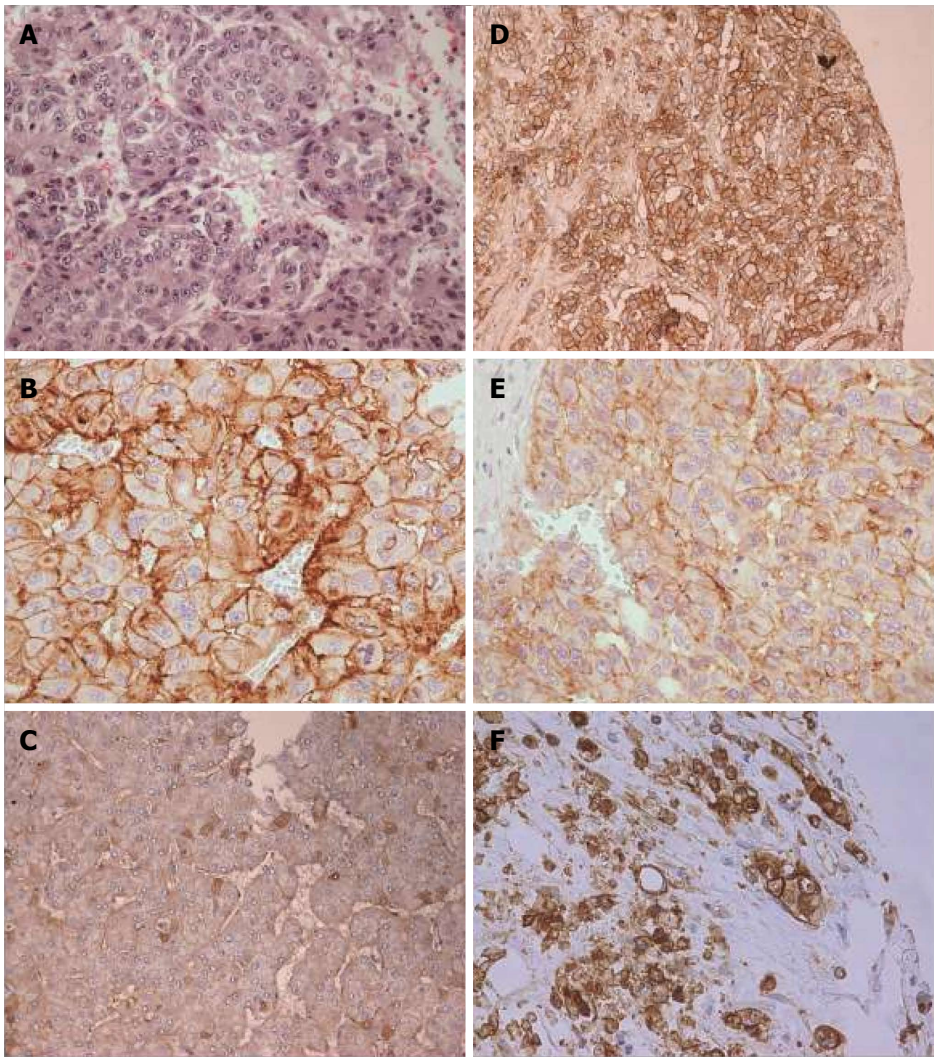

Figure 1 Representative positive immunoreactions. A: Hepatocellular carcinoma (HCC); B: Monocarboxylate transporter 1 (MCT1); C: MCT2; D: MCT4; E: CD147; F: Glucose transporter 1. Original magnification 400 ×.

Table 2 Association of overall expression of cell metabolism markers with the neoplastic (including metastasis) and non-neoplastic status of the liver *n* (%)

	MCT1			MCT2			MCT4			CD147			GLUT1		
	n	Positive	*P* value	*n*	Positive	*P* value	*n*	Positive	*P* value	*n*	Positive	*P* value	*n*	Positive	*P* value
Non-neoplastic	26	25 (96.2)		26	23 (88.5)		26	3 (11.5)		26	22 (84.6)		26	0 (0.0)	
Primary tumor	73	46 (63.0)		74	52 (70.3)		76	31 (40.8)		75	36 (48.0)		76	7 (9.2)	
Metastasis	16	9 (56.2)		16	6 (46.2)		17	11 (64.7)		17	10 (58.8)		17	5 (29.4)	
			0.003			0.02			0.001			0.005			0.007

MCT: Monocarboxylate transporter; GLUT: Glucose transporter.

Table 3 Association of the plasma membrane expression of cell metabolism markers with the neoplastic (including metastasis) and non-neoplastic status of the liver *n* (%)

	MCT1			MCT2			MCT4			CD147			GLUT1		
	n	Positive	*P* value	*n*	Positive	*P* value	*n*	Positive	*P* value	*n*	Positive	*P* value	*n*	Positive	*P* value
Non-neoplastic	26	25 (96.2)		26	0 (0.0)		26	1 (3.8)		26	20 (76.9)		26	0 (0.0)	
Primary tumor	73	40 (54.8)		74	9 (12.2)		76	29 (38.2)		75	32 (42.7)		76	4 (5.3)	
Metastasis	16	9 (56.2)		16	1 (7.7)		17	8 (47.1)		17	9 (52.9)		17	3 (17.6)	
			0.001			0.169			0.002			0.011			0.052

MCT: Monocarboxylate transporter; GLUT: Glucose transporter.

Table 4 Association of the plasma membrane expression of monocarboxylate transporters with the plasma membrane expression of the chaperone CD147 and the glycolytic protein glucose transporter 1 *n* (%)

		CD147			GLUT1	
	n	Positive	*P* value	*n*	Positive	*P* value
MCT1			< 0.001			1.000
Negative	40	4 (10.0)		40	2 (5.0)	
Positive	74	56 (75.7)		74	4 (5.4)	
MCT2			0.766			0.434
Negative	102	56 (54.9)		103	5 (4.9)	
Positive	10	5 (50.0)		10	1 (10.0)	
MCT4			0.114			0.004
Negative	80	46 (57.5)		81	1 (1.2)	
Positive	36	15 (41.7)		38	6 (15.8)	

MCT: Monocarboxylate transporter; GLUT: Glucose transporter.

of the markers were more highly expressed at the plasma membrane, with the exception of MCT2, which was expressed at a higher level in the cytoplasm. Importantly, expression of both MCT4 and GLUT1 were progressively increased from non-neoplastic to primary HCC, reaching a maximum frequency in metastases (Tables 2 and 3). In contrast, MCT2 expression was progressively decreased from non-neoplastic to primary HCC, reaching a minimum frequency in metastases (Table 3). MCT1 and CD147 were also significantly different among the hepatic lesion groups, showing a higher expression in the non-neoplastic samples compared to primary HCC and metastasis (Tables 2 and 3). Additionally, plasma membrane expression of MCT1, but not MCT4 or MCT2, was associated with plasma membrane expression of CD147 ($P < 0.001$; Table 4), while plasma membrane expression of MCT4 was associated with plasma membrane expression GLUT1 ($P = 0.004$; Table 4).

No significant association of the total expression or plasma membrane expression of MCTs with the clinical-pathological data was observed. Examinations of the relationships between total and plasma membrane expression of CD147 and GLUT1 with the clinical-pathological data have shown that the gender and architectural pattern of the lesions are important factors in these associations. MCTs, CD147 and GLUT1 were not associated with EGFR, Ki-67 and Keratin 19, which are important markers in a recently proposed molecular classification of HCC[18]. Associations of MCTs, CD147 and GLUT1 with the apoptosis marker caspase 3, were not significant as well. The exception was the significant correlation between MCT4 expression and Ki-67 counting. MCT1 expression, both in cytoplasm and plasma membrane, was higher in the absence of cirrhosis ($P = 0.044$ and $P = 0.015$, respectively). Cytoplasmic MCT2 positive staining was associated with liver weight lower than 1.5 kg ($P = 0.027$), absence of distant metastasis ($P = 0.036$) and absence of abdominal dissemination ($P = 0.010$), while plasma membrane MCT2, similar to MCT1, was associated with absence of cirrhosis ($P = 0.036$). Despite the fact that MCT4 did not correlate with the clinical-pathological variables assessed herein, MCT4 expression in the plasma membrane was strongly associated with Ki-67 positivity ($P = 0.004$) and also tended to associate with K19 expression ($P = 0.087$). CD147 plasma membrane expression was more frequent in HCC unrelated to cirrhosis ($P = 0.038$). Additionally, cytoplasmic ($P = 0.008$) and plasma membrane ($P = 0.005$) CD147 positive staining were associated with the absence of HBV infection. Finally, GLUT1 positive expression was higher in HCC affecting women ($P = 0.041$ for cytoplasmic positivity and $P = 0.033$ for membrane positive reaction).

DISCUSSION

The role of MCTs in tumor biology has been explored in recent years[3,6-9]. We have previously addressed MCTs expression in different malignancies compared to respective non-neoplastic parenchyma[3,6-9,10-12,20]. From those studies, we have learned that members of the MCT family may behave heterogeneously in each type of tumor and, especially MCT1 and/or MCT4, are highly expressed selected and, in some of them, are associated with worse prognostic markers[11,12,21]. Additionally, *in vitro* and *in vivo* evidence favors the hypothesis that some MCT isoforms might even be considered potential therapeutic targets[5,22,23].

The present study is the first to assess the possible role of MCTs in hepatocellular carcinoma. Our decision to approach our large autopsy series of HCC in this first study before assessing our series of biopsies or surgical specimens was based on the unique opportunity to compare MCT expression in non-neoplastic *vs* primary *vs* metastatic HCCs. However, inherent to autopsy studies, many of the tumors of the present series were advanced, thus reducing the impact of the assessment of clinical-laboratorial variables that might reflect initial steps of hepatocarcinogenesis. Because molecular integrity is not always assured in autopsy specimens, we did not perform mRNA studies, but the positive and negative controls performed herein assure that protein preservation was good enough to yield reliable immunohistochemical studies.

The most frequent patterns of HCC, as studied in the present necropsy series, show only a minor stromal component; therefore, in the current study, we did not particularly focus on stroma. Future studies should specifically address stromal-rich HCC subtypes, especially fibrolamellar HCC and sclerosing HCC.

The observed activity of MCTs 1, 2 and 4, CD147 and GLUT1 in HCC is partially aligned with most of our previous observations in adenocarcinomas from other organs[3]. Membrane expression of MCT4 and, less frequently, GLUT1 was found almost restricted to neoplastic liver and was higher in metastases. MCT4 expression was significantly related to a higher Ki-67 Ag index and also tended to associate with K19 expression (data not shown). Also, MCT4 was more frequent in higher histological grades (36.4% in g.1, 37.2% in g.2 and 63.6% in g.3), although this relationship was not statistically

significant. These relevant data link MCT4 activity with high proliferative index and possibly to the "progenitor cell component" of HCC, pointing to a role of MCT4 in HCC progression and/or more aggressive course.

As previous studies have extensively shown, the transition from high-grade dysplastic nodule to early HCC, as well as from "small, well-differentiated, indistinct border HCC" to "progressed HCC", runs in parallel with an abrupt shift in the vascular pattern[24]. We speculate that this fact could lead to important neo-angiogenesis that is immunohistochemically identified by diffusely CD34-positive microvessels, which might correspond to the shift to a preferably glycolytic pattern of energy source through the Warburg effect. At this point, MCT4 and GLUT1 would become overexpressed, in accordance with previous evidence that MCT4 is highly expressed in response to hypoxia, mostly mediated by HIF-1 α[3,25]. Interestingly, due to the double arterial and portal vein blood inflow, the liver is *per se* an organ normally exposed to "hypoxia", which is potentiated in cirrhosis and in malignant transformation[26]. In the cirrhotic liver, inflammation, hepatocytic lesion formation and regeneration, and neo-vessel formation are associated with the hypoxic setting, and stem cell activation is stimulated because these cells are able to resist this acidic/hypoxic microenvironment where cancer development and progression are strongly favored[24]. In livers chronically infected by HBV and HCV, the consequent cirrhosis is associated with a remarkable decrease in oxygen supply and, as such, the microenvironment induces stabilization of HIF-1α, which promotes angiogenesis by activating the transcription of vascular endothelial growth factor and cyclooxygenases and activating matrix metalloproteinases[26]. In summary, hypoxia induces cell damage and inflammation, limits liver regeneration and is strongly associated with HCC development[27]. Additionally, hypoxia was proved to be associated with glycolysis upregulation[28]. This result was partially demonstrated in the present study, as MCT4 and GLUT1 were more frequently expressed in primary HCC and metastasis than in non-neoplastic hepatocytes, thus corroborating previous evidence that GLUT1 is not detectable in normal epithelial tissues and benign epithelial neoplasms[28,29]. GLUT1 expression was previously demonstrated to be increased in HCC and promotes hepatic carcinogenesis[29].

MCT1 and its chaperone CD147, as well as MCT2, were found in this study to be more frequently expressed in non-neoplastic tissue than in HCC. Among the hepatocellular carcinomas, these proteins were significantly most commonly expressed in cases not related to HBV infection and in those occurring in non-cirrhotic livers. These observations warrant additional studies. This evidence points to the possible important role of MCT1 and CD147, and possibly MCT2, in the liver cell energy system. In contrast, MCT4 seems to be responsible for maintaining the glycolytic and acid resistant phenotype of cancer cells during the progression of HCC, with higher expression in advanced stages. In this context, it is important to highlight that while MCT4 affinity for the substrate makes this protein a transporter specialized in lactate efflux, MCT1 affinity for the substrate allows this isoform to promote both uptake and efflux of lactate. Therefore, the expression of MCT1, associated with its chaperone CD147, in non-neoplastic hepatic tissue may be related to one or both lactate transport directions, but most likely with the uptake of lactate because this tissue is a gluconeogenic tissue. Following this rationale, it was also expected to find plasma membrane expression of MCT2 (the isoform specialized for substrate uptake) in non-neoplastic hepatic tissue, as previously described for normal liver[25]. However, plasma membrane expression of MCT2 was not found in non-neoplastic tissue. Instead, MCT2 was highly expressed in the cytoplasm of non-neoplastic tissue and progressively decreased towards metastasis. This important cytoplasmic expression of MCT2 might be related to mitochondrial pyruvate transport, as MCTs were already described to be present in the mitochondria[30]. Because the metabolic behavior of liver cells in cancer will be adapted towards glucose consumption instead of glucose production, the expression of MCT2 will no longer be required, explaining the progressive decrease of MCT2 expression in the cytoplasm. In fact, the portion of tumors that maintains MCT2 expression in the cytoplasm should have a metabolism more similar to that found in normal tissues and therefore be less aggressive. This result explains the association between MCT2 cytoplasmic expression and variables related to a less aggressive profile, such as lower liver weight, absence of metastasis and absence of abdominal dissemination.

Interestingly, the increase in plasma membrane expression of MCT4 was not accompanied by CD147, similar to what was observed and expected for MCT2, but in contrast to what was observed for MCT1 (since, as expected, MCT1 was co-expressed with CD147). This information points to the existence of another MCT chaperone, as already hypothesized by our group in other studies[20]. Therefore, further studies will be important to search for the additional MCT chaperone, as it is essential for MCT activity and may also be regulating its expression. It is important to emphasize that MCTs and their chaperones are predominantly expressed in plasma membrane, their natural location, in both normal and neoplastic cells. Thus, it is not surprising that the hyper-expression of MCTs and CD147 corresponded more consistently with clinical data in different solid tumors[3].

In conclusion, our results assessed for the first time the role of MCTs in the liver and in HCC. In the present autopsy series, mostly representative of advanced HCC, MCT4 and GLUT1 were progressively highly expressed from non-neoplastic to primary HCC to metastatic hepatocellular carcinoma, in contrast to MCT2 that decreased towards malignancy and was associated with less advanced tumors. In addition to pointing to an important role of the hypoxia pathways in the progression of HCC, these data might add HCC to the list of possible

beneficiaries of anti-MCT therapies. Moreover, MCT4 was strongly associated with augmented Ki-67 expression, showing also a relevant trend to association with the "progenitor-cell component-related" keratin 19 and to higher histological grades. Future studies should further assess morphologically and molecularly the interactions related to the hypoxia and metabolic pathways in the development of HCC, especially approaching surgical samples representative of the sequence cirrhosis - dysplastic nodules - HCC.

COMMENTS

Background

To concisely and accurately summarize the related background of the article and to enable the readers to gain some basic knowledge relevant to the article, thus helping them better understand the significance of the article.

Research frontiers

To briefly introduce the hotspots or important areas in the research field related to the article.

Innovations and breakthroughs

To summarize and emphasize the differences, particularly the advances, achievements, innovations and breakthroughs, from the other related or similar articles so as to allow the readers to catch up the major points of the article.

Applications

To summarize the actual application values, the implications for further application and modification, or the perspectives of future application of the article.

Terminology

To concisely and accurately describe, define or explain the specific, unique terms that are not familiar to majority of the readers, but are essential for the readers to understand the article.

Peer review

To provide the comments from peer reviewers that most represent the characteristics, values and significance of the article, and allow the readers to have an objective point of view toward the article.

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PRSS1 and *SPINK1* mutations in idiopathic chronic and recurrent acute pancreatitis

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Abstract

AIM: To identify gene mutations in *PRSS1* and *SPINK1* in individuals with early onset idiopathic chronic or recurrent acute pancreatitis.

METHODS: The cationic trypsinogen gene (*PRSS1*; exons 2 and 3) and the serine protease inhibitor Kazal 1 gene (*SPINK1*; exon 3) were selectively amplified and sequenced from blood samples of 19 patients admitted to the Pancreas Clinic at our institution with chronic pancreatitis and/or idiopathic recurrent acute pancreatitis that were diagnosed or with onset before age 35. Fifty healthy volunteers served as controls. Whole blood samples were collected and gene specific sequences were amplified by polymerase chain reaction (PCR). All PCR products were subsequently sequenced in order to identify the presence of any mutations.

RESULTS: Nineteen patients with pancreatitis (14 males; median age 24 years, range 15-48 years) were included in this study, of which five showed the presence of gene mutations. Direct sequencing results indicated the presence of two previously unidentified mutations in exon 2 of *PRSS1* (V39E and N42S) in two patients with recurrent acute pancreatitis. Two cases had the N34S *SPINK1* mutation. Analysis of the relatives of one patient homozygous for this mutation showed that five of the six family members carried the N34S *SPINK1* mutation. Of these members, three were healthy heterozygous carriers and two were homozygotes (one sibling had diabetes, the other was healthy). Another patient was heterozygous for a novel *SPINK1* mutation located on exon 3 (V46D). All members from this patient's family had normal genotypes, indicating that it was a *de novo* mutation. No mutations in either gene were present in the control subjects.

CONCLUSION: Two novel *PRSS1* mutations and one novel *SPINK1* mutation were identified in Mexican patients with early onset idiopathic recurrent acute pancreatitis.

Key words: Cationic trypsinogen; *SPINK1*; *PRSS1*; Chronic pancreatitis; Recurrent acute pancreatitis; Hereditary pancreatitis

Core tip: Chronic and recurrent idiopathic pancreatitis has been associated with mutations in genes responsible for the synthesis of pancreatic proteases (*PRSS1*) and protease inhibitors (*SPINK1*). The distribution of these mutations varies among countries, but has not been examined in detail in Latin American countries. This study examined *PRSS1* and *SPINK1* in 19 Mexican subjects with chronic pancreatitis and/or idiopathic recurrent acute pancreatitis and identified two novel *PRSS1* mutations and one novel *SPINK1* mutation.

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INTRODUCTION

Chronic pancreatitis (CP) is a progressive inflammatory disease that leads to fibrosis and different degrees of exocrine and/or endocrine insufficiency[1]. There are many factors contributing to disease development, including alcohol use[2], though some cases do not present any known risk factors and are classified as idiopathic. Hereditary pancreatitis is diagnosed in the case of a positive family history[3]. As early as 1952, the observation that CP clustered in certain families suggested a genetic component. However, identification of such genetic factors did not occur until 1996, when mutations in the cationic trypsinogen gene (*PRSS1*) were discovered in families with hereditary CP and in some cases of idiopathic CP[4,5]. Later, mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*)[6,7], trypsin inhibitor (*SPINK1*), and chymotrypsinogen C (*CTRC*) genes were described in both idiopathic CP and alcoholic CP[8,9].

PRSS1 mutations have been linked with hereditary pancreatitis, whereas mutations in the *SPINK1* gene have been associated with pancreatitis of different etiologies[10]. However, mutations in *SPINK1* are not always sufficient to induce pancreatitis, and additional pancreatitis-associated factors must be present in order to express the disease. For example, the commonly observed N34S mutation in *SPINK1* by itself has no apparent functional effect[11,12]. *CTRC* mutations have been identified in patients with idiopathic CP and hereditary pancreatitis[13,14], as well as in subjects with alcoholic CP. The effect of these genes on pancreatitis likely results from an imbalance between normal mechanisms of protease activation and inhibition and pancreatic fluid composition[15,16]. Functional analysis of several identified gene mutations has shown that they result either in a gain of trypsin function (*PRSS1*)[17,18], loss or decreased protein expression or function (*SPINK1* D50E and Y54H)[19,20], and/or altered ductal secretion (*CFTR* mutations)[21].

The distribution of these identified mutations varies among countries[22-25]; although, reports from Latin America are scarce, with only information from Brazil available[26,27]. Furthermore, there is no available information about the role and characteristics of CP-related genetic mutations in Mexico, a population characterized by a broad genetic admixture[28]. However, a previous study by our group found that a large proportion of CP cases in Mexico are idiopathic[29]. Therefore, the aim of the present study was to identify mutations in the *PRSS1* and *SPINK1* genes in Mexican subjects with early onset idiopathic CP or idiopathic recurrent acute pancreatitis (IRAP).

MATERIALS AND METHODS

Subjects with CP and/or IRAP that were diagnosed or with onset before age 35 were prospectively and retrospectively enrolled in the study. For retrospective enrollment, the outpatient and inpatient database from the Pancreas Clinic at our institution was searched, and all eligible subjects were contacted by telephone. For prospective enrollment, all consecutive patients seen at our institution either as inpatients or at the outpatient Pancreas Clinic for CP or IRAP were included. Informed consent was obtained from the patients and 50 healthy volunteers who agreed to participate, and 20 cc of whole blood samples were then collected by peripheral vein puncture. Blood was stored at -70 ℃ for subsequent DNA extraction. This study was approved by the Institutional Review Board of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán.

Definitions

Acute pancreatitis was defined by the presence of two of the following: typical abdominal pain, three-fold elevation of serum pancreatic enzymes (amylase and/or lipase), and imaging evidence of acute pancreatitis. CP was considered if patients had any of the following predetermined criteria: histologic evidence (when available), imaging evidence from endoscopic retrograde cholangiopancreatography and/or magnetic resonance cholangiopancreatography with definitive evidence of CP according to the Cambridge classification, presence of pancreatic calcifications on computed tomography scan, plain abdominal X-rays, five or more CP-related findings on endoscopic ultrasound, and definitive pancreatic exocrine insufficiency according to a pancreolauryl test. IRAP was defined as the presence of two or more attacks of documented acute pancreatitis with no evident etiology after a thorough work-up and without imaging evidence of CP.

DNA extraction and gene- and exon-specific amplification

Whole blood (20 cc) was collected in K2EDTA BD Vacutainer tubes (Beckton Dickinson and Company, Franklin Lakes, NJ, United States). All blood specimens were processed at the Genomic Medicine Unit at the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán. Genomic DNA was extracted using a standard salt precipitation protocol and assessed for quality and quantity by agarose gel electrophoresis. Exons 2 and 3 of the *PRSS1* gene were amplified using primers and PCR amplification protocols previously reported by Nishimori *et al*[30] and Pho-Iam *et al*[31], respectively. Exon 3 of the *SPINK1* gene was amplified using primers and polymerase chain reaction (PCR) amplification protocols as previously reported by Witt *et al*[8].

DNA sequencing

The PCR products from all samples were purified using a QIAquick PCR Purification Kit (Qiagen, Venlo, Limburg, The Netherlands) according to the manufacturer's proto-

Table 1 Clinical characteristics of subjects with early onset chronic pancreatitis and/or idiopathic recurrent acute pancreatitis

Patient case No.	Sex	Age, in years	Age at symptom onset or diagnosis, in years	Clinical presentation	Mutation
1	Female	45	8	IRAP, pancreatic calcifications at age 15, Puestow procedure at age 22	V39E PRSS1
6	Male	26	20	IRAP, pancreatic calcifications at age 22	N42S PRSS1
8	Female	26	26	IRAP, pancreatic calcifications and dilated main pancreatic duct	N34S SPINK1
15	Male	20	16	IRAP, pancreatic calcifications	V46D SPINK1
19	Male	15	12	Abdominal pain, jaundice, pancreatic calcifications	N34S SPINK1

IRAP: Idiopathic recurrent acute pancreatitis.

col. Gene sequencing was performed using the Applied Biosystems Inc. (ABI) Prism BigDye Terminator Ready Reaction Cycle Sequencing Kit and the ABI Prism DNA Sequencer (model PE; ABI of Thermo Fisher Scientific, Waltham, MA, United States). All PCR products were sequenced in both directions using the same primers that were employed for the PCR amplification.

RESULTS

Nineteen subjects with either early onset CP or IRAP were identified and agreed to participate (14 males; median age 24 years, range 15-48 years) with a total of five instances of *PRSS1*/*SPINK1* gene mutations (Table 1). Two mutations in *PRSS1* were found: V39E in case 1 and N42S in case 6, both of which were in exon 2. The N34S *SPINK1* mutation was identified in cases 8 and 19. The family of one of these patients, who was homozygous for the N34S mutation and had early onset chronic calcifying pancreatitis, was also examined, and six of the 14 additional members agreed to participate and provide blood samples for sequencing. Although no *PRSS1* mutations were found in these family members, five of the six carried the same N34S *SPINK1* mutation. Of these, both parents and one sibling were healthy and heterozygous for this mutation, whereas two siblings were homozygous (one was healthy and the other had developed diabetes at an early age). The other studied sibling had a normal genotype and was otherwise healthy. The fifth mutation was found in case 15, who was a heterozygote for a new *SPINK1* mutation located on exon 3 (V46D). The family members of this patient were also studied, though no *PRSS1* mutations were found, and the V46D or other *SPINK1* mutations were not present. Moreover, no *PRSS1* or *SPINK1* mutations were identified in samples from the 50 healthy controls.

DISCUSSION

The results of this study identified two previously undocumented *PRSS1* mutations. The first mutation, N42S, represents the substitution of one polar hydrophilic amino acid with another similar one. However, the second novel mutation identified, V39E, represents the substitution of a non-polar hydrophobic amino acid to a negatively charged polar one, which could induce a conformational change in the final synthesized molecule. Although functional studies are needed to elucidate the effect of these mutations on protein structure, expression and/or secretion as well as their contribution to the pathogenic mechanisms of pancreatic injury, the results from the current study suggest that mutations in the *PRSS1* gene are sufficient to induce pancreatic disease. Indeed, *PRSS1* gene mutations have been directly implicated in the pathophysiology of hereditary and idiopathic CP by producing an autolysis-resistant trypsin and/or facilitating auto-activation[17,18]. However, the presence and contribution of mutations in other exons, genes or environmental factors remains unclear and should not be ruled out.

Recent reports from India[22] and Japan[23] indicated that *SPINK1* mutations confer strong genetic susceptibility to developing CP, but alone do not cause the disease. Some *SPINK1* mutations alter peptide expression or binding affinity, though the disease-causing biochemical defect of the N34S mutation remains unknown[11,12,19,20]. In the current study, one case with the *SPINK1* N34S mutation had apparently unaffected family members, two healthy siblings who were homozygous for the same mutation, and another sibling and both parents who were heterozygous. Thus, *SPINK1* mutations may require other associated genetic and/or environmental risk factors in order to promote pathogenicity. It is possible that these mutations impact the phenotypic presentation of the disease, with patients developing CP at earlier ages[8,9,12], as seen in this early onset CP population.

The sequencing results of this study identified a novel *SPINK 1* mutation (V46D) in a patient with established calcific CP and no other evident predisposing factors. Computational simulations could indicate if this mutation likely aborts SPINK1 protein synthesis, in contrast to other previously described mutations that reduce the enzymatic activity[32]. It appeared to be a *de novo* mutation, as none of the family members had it nor did they present any clinical manifestations of pancreatitis, though no paternity tests were run. In addition, neither the family members nor the affected individual had prior history of exposure to pancreatic disease-related risk factors. As the presence of mutations in other exons or genes remains unknown, a direct causal effect of this new mutation needs to be validated.

In agreement with previous studies worldwide[24,25], the current study provides further support that the frequency, nature and type of mutations vary among populations. This is the first Mexican study to explore the genetics of

early onset idiopathic CP in Hispanics. Although still a minority in the United States[33] and European countries, the Hispanic population has shown a steady and continuous growth rate, and thus the results provide valuable information to health care workers responsible for the medical attention of such minorities. The main limitations of this study include the small sample population, incomplete sequencing of the entire *PRSS1* and *SPINK1* genes, and absence of testing for *CFTR* and *CTRC* mutations. However, the findings of this study are consistent with previous reports and identify new pancreatitis-related mutations.

CONCLUSION

Mexican subjects with idiopathic CP and IRAP present similar mutations in the *PRSS1* and *SPINK1* genes as reported in other populations. This study identified three novel mutations, two in *PRSS1* and one in *SPINK1*, which may be unique to the Mexican population. The novel V46D *SPINK1* mutation may play a direct causal role of pancreatitis, though this finding needs to be validated by future functional studies.

COMMENTS

Background

Early onset chronic pancreatitis and idiopathic recurrent acute pancreatitis in the absence of any other established risk factors might result from genetic mutations. Gene mutations that have been linked with pancreatitis result in gain of function or inability to inhibit trypsin, or alteration in secretory mechanisms of the pancreatic ductal cells. Hereditary pancreatitis is an autosomal dominant condition characterized by recurrent attacks of acute pancreatitis in childhood or adolescence which progresses to the development of chronic pancreatitis at early ages. The first reported associated mutation was identified in the cationic trypsinogen gene (*PRSS1*) on chromosome 7. Additional mutations that may contribute are found in the serine protease inhibitor Kazal type 1 (*SPINK1*), the cystic fibrosis transmembrane conductance regulator gene (*CFTR*), and the chymotrypsinogen C (*CTRC*) gene. Mutations in these latter genes are seen in forms of pancreatitis that are initially classified as idiopathic chronic or idiopathic acute pancreatitis, although *PRSS1* mutations have also been seen in non-hereditary cases. These mutations may have an additive effect, increasing individual susceptibility to pancreatitis.

Research frontiers

Prior reports indicate that new mutations do occur across populations. Due to the genetic heterogeneity, screening for known and new mutations and characterizing them in each population is worthwhile.

Innovations and breakthroughs

This report identifies three new mutations, one in *SPINK1* and two in *PRSS1*, which are associated with chronic pancreatitis and may be unique to the Mexican population. These data suggest that there are wide genetic and population heterogeneities of the disease.

Applications

Chronic pancreatitis increases the risk of pancreatic cancer, and hereditary pancreatitis has an estimated cumulative risk of pancreatic cancer near 40%. Although there are no specific treatment recommendations in patients carrying pancreas-related mutations, identification of such could benefit genetic counseling, which is not used for other forms of pancreatitis, and result in the implementation of individualized and specific screening strategies for pancreatic cancer as well as lifestyle recommendations and modifications. In addition, the identification of these gene mutations will decrease the incidence and prevalence of idiopathic pancreatitis.

Terminology

Cationic trypsinogen, encoded by the *PRSS1* gene, represents 60% of the trypsinogen secreted by pancreatic acinar cells. Trypsinogen is then converted to trypsin by enterokinase within the duodenum, which then activates the digestive enzyme cascade. Pancreatic secretory trypsin inhibitor, or serine protease inhibitor Kazal type 1, is a protein encoded by the *SPINK1* gene that competitively binds to and inactivates trypsin.

Peer review

The present study provides new information concerning genetic contributors to chronic pancreatitis in the Mexican population, which has been largely unstudied to date. Patients and relatives were sampled to allow for direct sequencing to promote an understanding of the impact of the occurrence of identified mutations in the development of pancreatitis. The inclusion criteria were restricted to the defined characteristics of an uncommon disease, allowing for the selection of patients most likely to have relevant genetic mutations.

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CASE CONTROL STUDY

Molecular detection of monocyte chemotactic protein-1 polymorphism in spontaneous bacterial peritonitis patients

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Abstract

AIM: To investigate the association of the functional monocyte chemotactic protein-1 (*MCP-1*) promoter polymorphism (A-2518G) with spontaneous bacterial peritonitis (SBP).

METHODS: Fifty patients with post-hepatitis C liver cirrhosis and ascites were categorized into two groups; group Ⅰ included 25 patients with SBP and group Ⅱ included 25 patients free from SBP. In addition, a group of 20 healthy volunteers were included. We assessed the *MCP-1* gene polymorphism and gene expression as well as interleukin (IL)-10 levels in both blood and ascitic fluid.

RESULTS: A significant *MCP-1* gene polymorphism was detected in groups Ⅰ and Ⅱ (P = 0.001 and 0.02 respectively). Group Ⅰ was associated with a significantly higher frequency of AG genotype [control 8 (40%) *vs* SBP 19 (76.0%), P < 0.001], and group Ⅱ was associated with a significantly higher frequency of GG genotype when compared to healthy volunteers [control 1 (5%) *vs* cirrhotic 16 (64%), P < 0.001]. Accordingly, the frequency of G allele was significantly higher in both groups (Ⅰ and Ⅱ) [control 10 (25%) *vs* SBP 27 (54%), P < 0.001 and *vs* cirrhotic 37 (74.0%), P < 0.001, respectively]. The total blood and ascetic fluid levels of IL-10 and *MCP-1* gene expression were significantly higher in group Ⅰ than in group Ⅱ. Group Ⅰ showed significant reductions in the levels of *MCP-1* gene expression and IL-10 in the whole blood and ascetic fluid after therapy.

CONCLUSION: *MCP-1* GG genotype and G allele may predispose HCV infected patients to a more progressive disease course, while AG genotype may increase the susceptibility to SBP. Patients carrying these genotypes should be under supervision to prevent or restrict further complications.

Key words: Monocyte chemotactic protein-1; Genotype; Spontaneous bacterial peritonitis; Liver cirrhosis; Ascites; Gene expression; Interleukin-10

Core tip: Monocyte chemotactic protein-1 (*MCP-1*) polymorphism was investigated in hepatitis C virus (HCV) infected patients because of the higher susceptibility of cirrhosis and ascites patients to bacterial infections. MCP-1 secretion is up-regulated during chronic hepatitis and correlates with the severity of hepatic inflammation. Inheritance of *MCP-1* GG genotype and *MCP-1* G allele may predispose HCV infected patients to a more progressive disease course, while AG genotype may be a risk factor for spontaneous bacterial peritonitis (SBP) in patients with decompensated post-hepatitis C cirrhosis. MCP-1 expression and elevated IL-10 levels may be related to the development of SBP. HCV cirrhotic and SBP patients carrying the above genotypes should be under supervision and monitoring.

Salama MK, Sabry D, Al-Ghussein MAS, Ahmed R, AbdAllah S, Taha FM, Fathy W, Wadie MS, Nabih M, Abul-Fotouh A, Darwish T. Molecular detection of monocyte chemotactic protein-1 polymorphism in spontaneous bacterial peritonitis patients. *World J Gastroenterol* 2014; 20(33): 11793-11799 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i33/11793.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i33.11793

INTRODUCTION

Patients with cirrhosis and ascites show higher susceptibility to bacterial infections, mainly because of the inadequate defense mechanisms[1-3]. Factors influencing the development of spontaneous bacterial peritonitis (SBP) in patients with liver cirrhosis are poorly understood. Previous studies have indicated that peritoneal macrophages of cirrhotic patients might contribute to the control of SBP or influence its associated pathology in human cirrhosis by producing high quantities of angiogenic peptides and nitric oxide[4,5]. SBP can be caused by many reasons due to the alterations of the immune system that are very common in patients with end-stage liver disease and associated with an increased risk of infection and death[6,7]. Consequently, elevated concentrations of pro-inflammatory cytokines are found in ascitic fluid of these patients[8,9]. In addition, hepatitis C virus (HCV) infection is associated with increased hepatic expression of monocyte chemotactic protein-1 (MCP-1)[10].

MCP-1 acts as a chemotactic factor for monocytes/macrophages, activated lymphocytes and neutrophils during infections[11,12]; thus, these cells migrate to the ascitic fluid. Monocytes and macrophages release TNF-α and other cytokines, which in turn induce the expression of adhesion molecules on endothelial cells, thereby mediating a systemic reaction to the infection[11,12]. TNF-α has been shown to be elevated in the ascitic fluid of SBP patients, stimulating the release of interleukin-8 (IL-8), growth-related oncogene-α (GRO-α), and MCP-1 by mononuclear cells or endothelial cells. This release propagates the inflammatory reaction[13]. MCP-1 secretion is up-regulated during chronic hepatitis and correlates with the severity of hepatic inflammation[14,15]. Furthermore, a previous study showed elevated MCP-1 levels in ascitic fluid of cirrhotic patients with SBP compared to patients without SBP[13].

The aim of this work was to study the association of the functional *MCP-1* promoter polymorphism (A-2518G) with SBP and investigate the expression of MCP-1 in blood and ascites as well as serum and ascitic IL-10 levels.

MATERIALS AND METHODS

The case-control study protocol was performed in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. After being approved by the Institutional Review Board of Kasr El-Aini Hospital, the present study was conducted on 50 patients with post-hepatitis C liver cirrhosis and ascites attending the Kasr El-Aini Cairo University Hospital from February 2012 to September 2012. The study population is illustrated in Figure 1. Patients were categorized into two groups according to the presence of SBP or not as follows; group Ⅰ (n = 25) included patients with SBP proved by ascitic fluid polymorphonuclear leukocyte (PMN) count ⩾ 250 cells/mm^3, and group Ⅱ (n = 25) included patients without SBP. Patients with alcoholic liver cirrhosis, Wilson's disease, hemochromatosis, glycogen storage disease and malignant or tuberculous ascites were excluded from this study. As an additional control group (group Ⅲ), 20 healthy volunteers (15 males and 5 females) with a mean age of 48.28 ± 4.56 years were included in the study, and they were recruited from the members of the Medical Biochemistry Department, Faculty of Medicine.

Written informed consent to participate in the study was obtained from all participants. After that, they were subjected to a detailed medical history assessment and laboratory investigation (complete blood count, liver and renal function tests). Serum IL-10 level assessment, quantitative assessment of *MCP-1* gene expression in blood and detection of *MCP-1* gene polymorphism were performed. The ascitic fluid of patients of both groups Ⅰ and Ⅱ was analysed for IL-10 level and the quantitative assessment of *MCP-1* gene expression. Appropriate antibiotic medication therapy was prescribed for patients of group Ⅰ and after the ascitic fluid PMN count became less than 250 cells/mm^3, they were reassessed by measuring the *MCP-1* gene expression in the whole blood and in the ascitic fluid in addition to the IL-10 level in both serum and ascitic fluid.

Detection of MCP-1 polymorphism

Genomic DNA was prepared from venous blood sam-

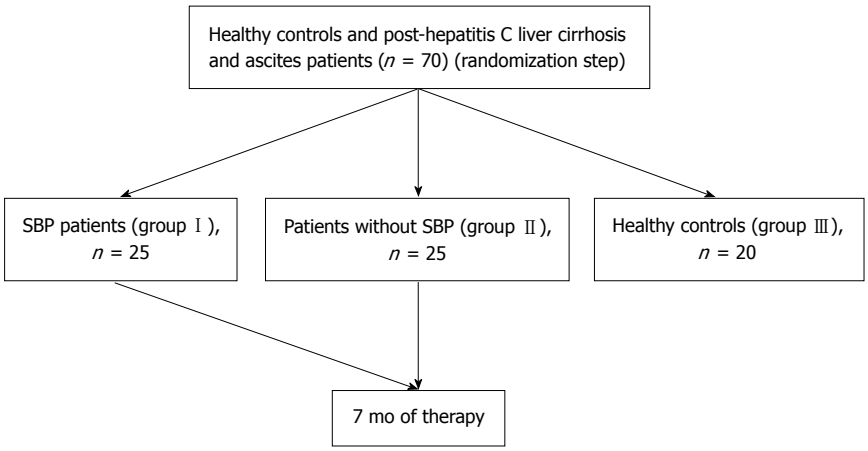

Figure 1 Algorithm for the study design. SBP: Spontaneous bacterial peritonitis.

ples using the Innu PREP blood DNA mini kit (Analytic Jena, Germany) following the manufacturer's instructions. The identification of the polymorphism was carried out using polymerase chain reaction (PCR), followed by a restriction fragment length polymorphism (RFLP) assay, using a PvuII site, which is introduced by the presence of the G nucleotide. The regulatory region of the *MCP-1* gene (from -2746 at -1817) was amplified by PCR using a forward primer (5'-CCGAGATGTTCCCAGCACAG-3') and a reverse primer (5'-CTGCTTTGCTTGTGCCTCTT-3')[16].

PCR was performed in a 40 μL reaction system containing 10 × buffer (10 mmol/L Tris-HCl pH 9, 2.0 mmol/L $MgCl_2$, 50 mmol/L KCl), 200 μm dNTPs, 2.5 pmole of each primer, 5 μL of DNA, 0.5 U Taq polymerase (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and ddH_2O. The following thermal profiles were run: 95 ℃ for 40 s, 56 ℃ for 30 s, and 72 ℃ for 4 min. After a final extension of 10 min at 72 ℃, 7 μL of the PCR products were resolved on 2% agarose gels and stained with ethidium bromide to visualize the expected 930-bp band. After visualization, 8 μL of the PCR products were digested with 10 U of PvuII in 10× buffer and H_2O up to a final volume of 20 μL at 37 ℃ for 2 h. The resulting products were separated by electrophoresis on 1.5% agarose gels, containing ethidium bromide at a final concentration of 0.5 g/mL. Samples showing only a 930-bp band were assigned as A/A, those showing two bands at 708 and 222 bp were considered G/G and those showing three bands at 930, 708 and 222 bp were typed as A/G.

Quantitative assessment of MCP-1 gene expression by real-time PCR

RNA extraction from blood and ascitic fluid samples: SV total RNA isolation system (Promega, USA) was used to extract RNA.

Primer design and selection: All primers were designed based on target sequences obtained from the reference[17].

cDNA synthesis: The extracted RNA was reverse transcribed into cDNA using RT-PCR kit (Stratagene USA)[18].

Real-time quantitative PCR using SYBR Green I

Real-time quantitative PCR (qPCR) amplification and analysis were performed using an Applied Biosystem with software version 3.1 (StepOne™, USA). The qPCR assay with the primer sets were optimized at the annealing temperature. All cDNA including previously prepared samples, internal control (for glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene expression as housekeeping gene), and non-template control (water to confirm the absence of DNA contamination in the reaction mixture), were in duplicate. The sequences of the primers used for amplification of the *GAPDH* gene were forward, 5'CGCTCTCTGCTCCTCCTGTT 3' and reverse, 5' CCATGGTGTCTGAGCGATGT 3'[19].

Estimation of serum and ascitic fluid IL-10 by enzyme linked immunosorbent assay

IL-10 was analysed using kits produced by Orgenium Laboratories Business Unit (Vantaa, Finland)[20].

Statistical analysis

The results were analysed using the SPSS software package version 9.0 (Chicago, IL, USA). Quantitative data are expressed as mean ± standard deviation (SD). Differences between two groups were compared by the Student's *t*-test. Genotype and allele frequencies were reported as percentages and the differences between groups were determined by χ^2 test. Correlations between data were performed using Pearson and Spearman correlation tests as required. Differences were considered significant at $P < 0.05$.

RESULTS

The demographic and clinical data of the studied groups are presented in Table 1. Patients of both groups and the healthy controls were age and sex matched. There was no statistically significant difference between both studied

Table 1 Baseline demographic and clinical characteristics of the studied groups

	Control group (n = 20)	Group Ⅰ (SBP) (n = 25)	Group Ⅱ (cirrhotic) (n = 25)
Age (yr)	48.28 ± 4.56	51.24 ± 9.3	47.08 ± 12.9
Sex (male)	15 (75)	18 (72)	15 (60)
BMI (kg/m^2)	28.42 ± 2.33	27.94 ± 2.1	28.2 ± 1.8
DM (Yes)	0	5 (20)	8 (32)
GIT bleeding (Yes)	0	5 (20)	5 (20)
Hepatic encephalopathy (Yes)	0	9 (36)	7 (28)
Duration of liver cirrhosis (yr)	0	4.46 ± 5	4.04 ± 3.26
Duration of ascites (yr)	0	1 ± 2.01	1.71 ± 1.54
Hemoglobin (g/dL)	12.6 ± 1.6	10.27 ± 1.95[b]	9.5 ± 2.22[b]
Platelets (10^3/μL)	158.4 ± 12.8	146.6 ± 90.2	118.6 ± 35.9[b]
TLC (10^3/μL)	6.3 ± 0.97	5.82 ± 3.05	6.7 ± 2.58
Serum albumin (g/dL)	4.34 ± 0.62	2.26 ± 0.39[b]	2.3 ± 0.46[b]
Total bilirubin (mg/dL)	1.036 ± 0.064	5.42 ± 8.7[b]	2.69 ± 2.65[b]
Direct bilirubin (mg/dL)	0.176 ± 0.078	3.07 ± 5.3[b]	1.44 ± 1.58[b]
Urea (mg/dL)	17.4 ± 3.3	59.45 ± 26.6[a,b]	42.8 ± 24.49[b]
Creatinine (mg/dL)	0.86 ± 0.208	1.96 ± 1.79[b]	1.5 ± 0.95[b]
AST (IU/L)	47.96 ± 7.7	69.86 ± 38.03[a]	85.8 ± 52.99[b]
ALT (IU/L)	23.79 ± 7.5	39.7 ± 16.03[b]	40.17 ± 24.76[a]
ALP (IU/L)	95.5 ± 19.8	167.9 ± 69.49[b]	101.4 ± 39.67
INR	0.996 ± 0.13	1.84 ± 0.59[b]	1.66 ± 0.40[b]
MCP-1 gene expression in whole blood	0.131 ± 0.0367	1.04 ± 0.119[b,d]	0.112 ± 0.046
Serum IL-10 (pg/mL)	14.48 ± 3.29	29.26 ± 7.037[b,d]	15.91 ± 4.53
PMN count in ascites (cells/mm^3)	-	1194.6 ± 1187.6[d]	110.3 ± 60.89
Serum-Ascites Albumin gradient (SAAG) (g/dL)	-	1.34 ± 0.107[d]	1.67 ± 0.32
Ascitic IL-10 (pg/mL)	-	60.07 ± 12.67[d]	16.86 ± 5.2
Ascitic *MCP-1* gene expression	-	2.251 ± 1.039[d]	1.5 ± 0.59

[a]P < 0.05, [b]P <0.01 *vs* control group; [d]P <0.01, group Ⅰ *vs* group Ⅱ. Results are expressed as mean ± SD or frequency (%) as required. BMI: Body mass index; DM: Diabetes mellitus; GIT: Gastrointestinal; TLC: Total lymphocyte count; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; INR: International normalized ratio; MCP-1: Monocyte chemotactic protein-1; SBP: Spontaneous bacterial peritonitis.

Table 2 *MCP-1* genotypes in the studied groups

	Control group (n = 20)	Group Ⅰ (SBP) (n = 25)	Group Ⅱ (cirrhotic) (n = 25)
Genotype			
AA	11 (55)	2 (8.0)[b]	4 (16)[b]
AG	8 (40)	19 (76.0)[b]	5 (20)[b,d]
GG	1 (5)	4 (16.0)	16 (64)[b,d]
Allele			
A	30 (75.0)	23 (46.0)[b,d]	13 (26.0)[b]
G	10 (25.0)	27 (54.0)[b]	37 (74.0)[b,c]

[a]P < 0.05, [b]P <0.01 *vs* control group; [c]P < 0.05, [d]P <0.01, group Ⅰ *vs* group Ⅱ. Results are expressed as frequency (%). MCP-1: Monocyte chemotactic protein-1.

groups of patients regarding the studied laboratory data, except for significantly higher levels of the *MCP-1* gene expression in whole blood (cirrhotic 0.112 ± 0.046 *vs* SBP 1.04 ± 0.119, P < 0.001) and serum IL-10 in SBP patients (cirrhotic 15.91 ± 4.53 *vs* SBP 29.26 ± 7.037, P < 0.001).

MCP-1 polymorphism in all the studied groups is presented in Table 2. Our results showed that the genotype frequencies in the healthy controls did not depart from those expected on the basis of Hardy-Weinberg equilibrium (P = 0.76). However, in cirrhotic patients without SBP (group Ⅱ) and those with SBP (group Ⅰ), the observed and expected frequencies were significantly different (P = 0.02 and 0.001, respectively). When compared to normal healthy volunteers, a significantly higher frequency of the GG genotype was reported in cirrhotic patients without SBP (group Ⅱ) [control 1 (5%) *vs* cirrhotic 16 (64%), P < 0.001], while a significantly higher frequency of the AG genotype was reported with cirrhotic patients with SBP (group Ⅰ) [control 8 (40%) *vs* SBP 19 (76.0%), P < 0.001]. When comparing the two groups of patients with each other, a significantly higher frequency of the GG genotype was reported with cirrhotic patients without SBP (group Ⅱ) [SBP 4 (16%) *vs* cirrhotic 16 (64%), P < 0.001], while a significantly higher frequency of the AG genotype was reported with cirrhotic patients with SBP (group Ⅰ) [SBP 19 (76.0%) *vs* cirrhotic 5 (20%), P <0.001]. Accordingly, there was a significantly higher frequency of the G allele in both groups of patients (Ⅰ and Ⅱ) when compared to healthy volunteers (control 10 (25%) *vs* SBP 27 (54%), P < 0.001 and *vs* cirrhotic 37 (74.0%), P < 0.001 respectively). When comparing both groups of patients with each other, it was revealed that the G allele represented 54% in those with SBP (group Ⅰ) *vs* 74% in those without SBP (group Ⅱ) [SBP 27 (54.0%) *vs* cirrhotic 37 (74%), P < 0.001], while the A allele represented 46% in those with SBP (group Ⅰ) *vs* 26% in those without SBP (group Ⅱ) [SBP 23(46.0%) *vs* cirrhotic 13 (26%), P < 0.001], and these differences were statistically significant.

Ascitic fluid analysis

Results are presented in Table 1. Our results revealed

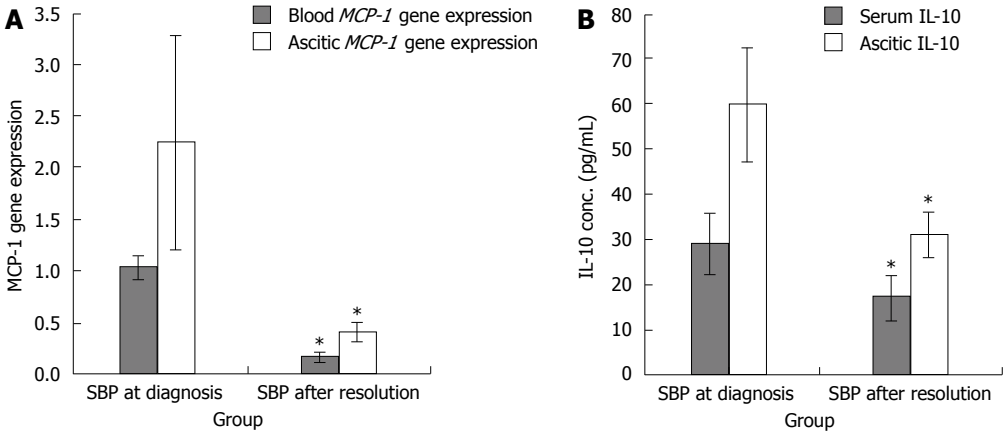

Figure 2 Cirrhotic patients with spontaneous bacterial peritonitis before and after therapy. A: Blood and ascitic *MCP-1* gene expression; B: Serum and ascetic IL-10 concentrations. Results are expressed as mean ± SD. Asterisk denotes a significant difference in measured parameters at diagnosis and after resolution. MCP-1: Monocyte chemotactic protein-1; IL-10: Interleukin 10; SBP: Spontaneous bacterial peritonitis.

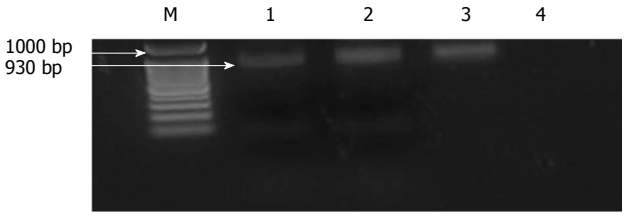

Figure 3 Agarose gel electrophoresis analysis of polymerase chain reaction products for *MCP-1* gene (930 bp) before digestion with restriction enzyme. Lane M: DNA ladder (100, 200, 300 to 1000 bp); Lane 1: polymerase chain reaction (PCR) product for *MCP-1* gene in a healthy control; Lane 2: PCR product for *MCP-1* gene in a cirrhotic patient with SBP; Lane 3: PCR product for *MCP-1* gene in a cirrhotic patient without SBP; Lane 4: Negative control. MCP-1: Monocyte chemotactic protein-1; SBP: Spontaneous bacterial peritonitis.

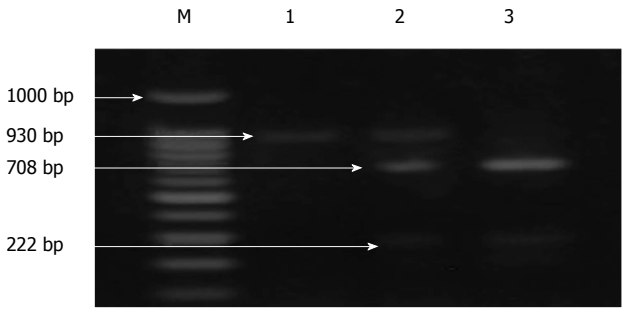

Figure 4 Agarose gel electrophoresis analysis of polymerase chain reaction products for *MCP-1* gene (930 bp) after digestion with restriction enzyme. Lane M: DNA ladder (100, 200, 300 to 1000 bp); Lane 1: polymerase chain reaction (PCR) product for A/A genotype (930 bp); Lane 2: PCR product for A/G genotype (930, 708 and 222 bp); Lane 3: PCR product for G/G genotype (708 and 222 bp). MCP-1: Monocyte chemotactic protein-1.

that the ascitic fluid levels of the *IL-10* and *MCP-1* gene expression were significantly higher in patients with SBP (group Ⅰ) than those without SBP (group Ⅱ).

Cirrhotic patients with SBP (group Ⅰ) showed significant reductions in the levels of *MCP-1* gene expression and IL-10 in the whole blood and ascitic fluid after therapy (Figure 2A and B). In cirrhotic patients with SBP a significant positive relationship was detected between the *MCP-1* gene expression in the whole blood and the duration of liver disease ($r = 0.46$, $P = 0.02$). Also, a significant positive relationship was detected between the serum IL-10 and both the SAAG and the serum albumin level ($r = 0.623$ and 0.472, $P = 0.023$ and 0.02, respectively). In addition, a significant positive relationship was detected between the ascitic *MCP-1* gene expression and the total bilirubin level ($r = 0.535$, $P = 0.03$). Contrarily, a significant negative relationship was detected between the ascitic *MCP-1* gene expression and the total leucocytic count (TLC) ($r = 0.671$, $P = 0.003$). However, these relationships were statistically insignificant in cirrhotic patients without SBP. On the other hand, a significant positive relationship was detected between the serum IL-10 and the urea level ($r = 0.449$, $P = 0.036$), as well as between the ascitic *MCP-1* gene expression and the serum creatinine level ($r = 0.57$, $P = 0.01$). A significant negative relationship was detected between the ascitic IL-10 and the duration of the liver cirrhosis ($r = 0.39$, $P = 0.048$). PCR products for *MCP-1* gene (930 bp) before digestion with restriction enzyme for different groups are shown in Figure 3. PCR products for *MCP-1* gene (930 bp) after digestion with restriction enzyme in Figure 4 showed A/A genotype at 930 bp, A/G genotype at 930, 708 and 222 bp and G/G genotype at 708 and 222 bp.

DISCUSSION

Interestingly, a significant *MCP-1* genotype polymorphism was observed in cirrhotic patients with and without SBP in our study, which was not observed in the healthy Egyptian volunteers. Further analysis showed that cirrhotic patients without SBP were associated with a higher frequency of GG genotype, while those with SBP were associated with a higher frequency of AG genotype. Also, it was found that the G allele frequency was significantly higher in both the cirrhotic patients with and without SBP than the healthy volunteers as well as being higher in the cirrhotic patients without SBP than in those with SBP. This result is in agreement with the finding by

Gäbele *et al*[21], who reported that carriers of the G allele of the *MCP-1* polymorphism were more frequent in patients with alcohol induced cirrhosis than in heavy drinkers without evidence of liver damage (controls). Also, in a previous study, carriers of the G allele were significantly more frequent in HCV patients with more advanced fibrosis and severe inflammation[14]. *In vitro* stimulated monocytes from individuals carrying a G allele at -2518 produced more *MCP-1* than cells from A/A homozygous subjects[22]. Carriers of the G allele were significantly more frequent in HCV patients with more advanced fibrosis and severe inflammation[15].

In patients with SBP, MCP-1 acts as a chemotactic factor for monocytes and macrophages; thus, these cells migrate to the ascitic fluid. These monocytes and macrophages release TNF-α and other cytokines, which in turn induce the expression of adhesion molecules on endothelial cells, thereby mediating a systemic reaction to the infection[11,12]. This explains the significant increase, reported in our study, of the mean level of the *MCP-1* gene expression in both blood and ascetic fluid of cirrhotic patients with SBP compared with cirrhotic patients without SBP, which was in agreement with previous studies[21-23]. These findings suggest that this potent chemokine plays a pathophysiological role during the development and the course of SBP. As well in our study, the SBP patients showed a significant increase in the mean level of PMN count compared with cirrhotic patients without SBP. In the present study, the mean level of *MCP-1* gene expression in blood was higher in control subjects than in cirrhotic patients without SBP. However, this difference was not statistically significant. This is in concordance with what was reported by Nischalke *et al*[24], who found that the MCP-1 was markedly lower in HCV-infected patients than in controls, and it was explained by the down-regulation of MCP-1 expression by viral proteins and the inhibition of activity of the *MCP-1* gene promoter by HCV core protein.

In agreement with the results of previous studies[13,25,26], our research reported a significant increase in the serum IL-10 level in the SBP patients than those in the healthy volunteers and cirrhotic patients without SBP. Also it was higher in cirrhotic patients without SBP than in healthy volunteers, but the difference was not statistically significant. This goes with the assumption that the elevated IL-10 levels in both cirrhotic patients with and without SBP have a regulatory role in the inflammatory process in liver cirrhosis patients[25].

Our study reported that mean level of serum ascites albumin gradient (SAAG) was significantly higher in cirrhotic patients than in SBP patients, and this is in agreement with the results of Khan *et al*[26] who found that the SAAG was higher in cirrhotic than SBP patients.

Changes in various cytokines levels after SBP treatment were previously observed, *e.g.*, MCP-1 and IL-10 levels showed a significant decrease during follow-up after treatment[13], and this is in agreement with the result of our study that SBP patients showed significant decreases in the mean levels of blood and ascitic fluid *MCP-1* gene expression and serum IL-10 after SBP treatment.

In conclusion, inheritance of *MCP-1* GG genotype and *MCP-1* G allele may predispose HCV infected patients to a more progressive disease course, while AG genotype may be a risk factor for SBP in patients with decompensated post-hepatitis C cirrhosis. MCP-1 expression and IL-10 levels in blood and ascitic fluid may be related to the development and the course of SBP. Further randomized controlled trials with greater sample size are recommended.

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COMMENTS

Background

The high susceptibility of hepatitis C patients with cirrhosis and ascites to bacterial infections correlates with peritoneal macrophages that might contribute to the control of spontaneous bacterial peritonitis (SBP) or influence its associated pathology. The chemotactic factor monocyte chemotactic protein-1 (MCP-1) secretion is up-regulated during chronic hepatitis and correlates with the severity of hepatic inflammation, and thus the functional *MCP-1* promoter polymorphism (A-2518G) can be associated with cirrhosis and SBP.

Research frontiers

The functional *MCP-1* promoter polymorphism (A-2518G) genotypes distribution and allele frequencies were demonstrated as markers for cirrhosis and/or SBP susceptibility in HCV patients. Above and beyond, MCP-1 expression and level along with IL-10 level were evaluated as pre- and post-treatment monitoring indicators for such cases.

Innovations and breakthroughs

Several reports have highlighted that carriers of the G allele of the MCP-1 polymorphism were more frequent in patients with alcohol induced cirrhosis and HCV fibrosis and severe inflammation. This is the first study to report that inheritance of *MCP-1* GG genotype and *MCP-1* G allele may predispose HCV infected patients to cirrhosis, while AG genotype may be a risk factor for spontaneous bacterial peritonitis SBP in patients with decompensated cirrhosis. Additionally, our investigations would propose MCP-1 expression and IL-10 levels in blood and ascitic fluid to be correlated with the development and the course of SBP.

Applications

HCV infected patients carrying the G allele of the *MCP-1* polymorphism should be under intensive observation, because *MCP-1* GG genotype carriers may develop cirrhosis and AG genotype can be a high risk factor for spontaneous bacterial peritonitis. MCP-1 expression and IL-10 levels in blood and ascitic fluid should be investigated during the development of these cases.

Terminology

MCP-1 is a signalling protein that acts as a chemotactic factor for monocytes and macrophages; thus, these cells migrate to the ascetic fluid. SBP is a peritoneal recurrent bacterial infection due to lower immunity state.

Peer review

This paper has high scientific and methodological levels.

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CASE CONTROL STUDY

Clinical presentations of gastric small gastrointestinal stromal tumors mimics functional dyspepsia symptoms

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Abstract

AIM: To explore whether clinical presentations of gastric small gastrointestinal tumors (GISTs) mimics gastrointestinal dyspepsia symptoms.

METHODS: The endosonographic data of 167 patients who underwent endoscopic submucosal dissection at the Tianjin Medical University General Hospital, China between 2009 and 2011 were analyzed. GISTs and leiomyomas had a similar intragastric distribution and similar locations within the gastric wall. Therefore, patients with GISTs were chosen as the study group and those with leiomyomas were chosen as the control group. Dyspepsia symptom questionnaires were used to investigate and compare the gastrointestinal symptoms of patients with GISTs and those with gastric leiomyomas before and after endoscopic submucosal dissection (ESD). The questionnaires evaluated symptoms such as epigastric pain, heartburn, regurgitation, epigastric discomfort, nausea and vomiting, abdominal bloating, and eructation. Symptoms were assessed using a four-point scoring scale.

RESULTS: GISTs were the most common gastric submucosal lesion (67 cases, 40.12%), followed by leiomyomas (38 cases, 22.75%). Both groups were similar in terms of gender distribution (P = 0.49), intragastric location (P = 0.525), and originating layer within the gastric wall (P = 0.449), but leiomyomas were more commonly found in the proximal fundus (P < 0.05). Overall, 94.2% of the patients with small GISTs and 93.5% of those with gastric leiomyomas experienced some dyspepsia; however, total symptom scores were significantly lower in the GIST group than in the leiomyoma group (1.34 ± 1.27 *vs* 2.20 ± 1.70, P < 0.05). Each component of the symptom score demonstrated a statistically significant improvement in the GIST patients after ESD (P < 0.05), including epigastric pain (0.80 ± 0.90 *vs* 0.13 ± 0.46), heartburn (0.63 ± 1.08 *vs* 0.13 ± 0.41), regurgitation (0.55 ± 0.87 *vs* 0.22 ± 0.57), epigastric discomfort (0.70 ± 0.98 *vs* 0.32 ± 0.47), nausea and vomiting (0.27 ± 0.62 *vs* 0.05 ± 0.21), abdominal bloating (0.70 ± 0.90 *vs* 0.27 ± 0.49), and eructation (0.36 ± 0.61 *vs* 0.21 ± 0.46). For leiomyoma patients, symptoms such as heartburn, nausea, vomiting, and eructation improved after treatment; however, these improvements were not statistically significant (P > 0.05). Thus, the pathophysiology of dyspepsia symptoms may be different between the two groups.

CONCLUSION: Symptoms of gastric small GISTs may mimic those of functional dyspepsia. An alternative diagnosis should be considered in patients with functional dyspepsia and treatment failure.

Key words: Gastric small gastrointestinal stromal tumor; Gastric leiomyoma; Clinical presentation; Endoscopic ultrasonography

Core tip: We compared the clinical presentations and endosonographic characteristics of gastric small gas-

trointestinal stromal tumors (GISTs) and gastric leiomyomas. Specifically, we compared the change in the clinical presentations of these two groups before and after endoscopic submucosal dissection. We found that the symptoms of small GISTs may mimic those of functional dyspepsia, and that small gastric GISTs may produce more severe symptoms than gastric leiomyomas due to the different histological origins. This study is novel as there has been no report regarding the clinical symptoms of dyspepsia caused by small gastric GISTs.

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumor of the gastrointestinal tract[1], with an annual incidence of 12.7-14.5 cases per million[2,3]. GISTs occur throughout the gastrointestinal tract, but they are preferentially located in the stomach (60%-70%). Recently, micro-GISTs [also called GIST tumorlets and interstitial cell of Cajal (ICC) hyperplasia, ≤ 1 cm in size] have been found in 9.1%-35% of stomachs that are thoroughly examined after surgical removal or at the time of autopsy[4-7]. Therefore, the incidence of gastric micro-GISTs is higher than that of gastric clinical GISTs.

It is widely accepted that GISTs originate from the ICCs[8] or mesenchymal stem cells that can differentiate into ICCs[9]. ICCs are specialized cells in the gastrointestinal tract that generate the rhythmic electrical and contractile activity that exists from the stomach to the rectum, and mediate enteric motor neurotransmission[10]. ICCs are essential for gut peristalsis.

The symptoms of GISTs are variable and depend on the size, site, relationship with the gastrointestinal wall, and malignancy[11,12]. When tumors grow to a certain size, gastrointestinal bleeding may result from ulceration of the mucosal surface or abdominal pain may arise from the compression of surrounding tissues or organs. However, small tumors may be asymptomatic or only present with nonspecific gastrointestinal symptoms[12]. It has been reported that a 62-year-old female patient was diagnosed and unsuccessfully treated for irritable bowel syndrome (IBS) for 11 years and was eventually found to have an obstructing small-bowel GIST. After operation, all of her persistent gastrointestinal symptoms including abdominal pain, nausea, bloating and constipation disappeared[13]. The case suggested that GISTs that conserve the function of ICCs could alter the motility of the gastrointestinal tract, especially when the tumor is sufficiently small. The symptoms may mimic those of functional gastrointestinal disorders. However, there has been no report regarding the clinical symptoms of dyspepsia caused by small gastric GISTs.

This study was designed to assess the change in symptoms before and after endoscopic submucosal dissection (ESD) among gastric small GIST patients using a dyspepsia symptom questionnaire, and to explore whether some dyspepsia symptoms would be associated with gastric small GISTs - the neoplastic transformation of ICCs.

GISTs are one type of submucosal lesions, which arise from tissue under the epithelial layer and include leiomyomas, lipomas, and ectopic pancreas. Various submucosal lesions have different site characteristics, layer of origin, and appearance under endoscopic ultrasound (EUS). Thus, the endosonographic features of gastric submucosal lesions were investigated for choosing the appropriate control group, to explore whether other submucosal lesions would cause some dyspepsia symptoms.

MATERIALS AND METHODS

Diagnosis

A total of 167 patients with a diagnosis of gastric submucosal lesions underwent ESD at the Endoscopy Center of Tianjin Medical University General Hospital between September 2009 and December 2011. Prior to ESD, all the patients underwent EUS to determine tumor location within the gastric wall, size and morphology, sonographic characteristics, and tumor margins. After ESD, all tumor specimens were examined by two experienced pathologists. Immunohistochemical analyses for CD117, CD34, smooth muscle actin, desmin, S-100 and DOG-1 were performed to determine the pathological diagnosis. GIST diagnosis met the criteria of the 2008 Chinese Consensus on the Diagnosis and Treatment of GIST[14]. The risk stratification of the GIST cases followed the 2008 National Institutes of Health (NIH) Consensus Classification System[15]. All patients voluntarily gave signed informed consent before ESD, and the study was approved by the Medical Review Ethics Committee of Tianjin Medical University.

The pathological diagnoses of the gastric submucosal lesions and their location in the stomach are shown in Table 1. GISTs were the most common gastric submucosal lesion (67 cases, 40.12%), followed by leiomyomas (38 cases, 22.75%). GISTs and leiomyomas had a similar intragastric distribution and similar locations within the gastric wall, as shown in Table 1. Therefore, patients with GISTs were chosen as the study group, and the patients with leiomyomas were chosen as the control group.

Study of gastrointestinal symptoms

Prior to ESD, a symptom questionnaire was obtained from each patient, who had undergone EUS with a diagnosis of gastric submucosal lesions at our endoscopy center between September 2009 and December 2011. The symptom questionnaire was completed by well-trained physicians in face-to-face interviews. Another symptom questionnaire was obtained from the patients

Table 1 Pathological diagnoses of gastric submucosal lesions and their location

	Gastric fundus			Gastric body			Gastric antrum		
	Muscularis mucosae	Submucosa	Muscularis	Muscularis mucosae	Submucosa	Muscularis	Muscularis mucosae	Submucosa	Muscularis
GIST (n = 67)			46			16	1		4
Leiomyoma (n = 38)			30			6			2
Neurilemmoma (n = 1)									1
Ectopic pancreas (n = 30)					1	2	8	13	6
Lipoma (n = 8)					3			5	
Carcinoid (n = 4)						1	1		2
Inflammatory fibrous polyps (n = 9)							1	8	
Others (n = 10)[1]						1	1	4	
Total	0	2	76	0	4	26	12	30	15

[1]Other lesions include vascular malformation, myoepithelial hamartoma, tuberculosis, lymphoma, and gastritis cystic profunda. GIST: Gastrointestinal tumor.

Table 2 Patient baseline features and endosonographic characteristics

	Leiomyoma (n = 38)	GIST (n = 67)	P value
Gender			
Male	15	30	0.597
Female	23	37	
Age (yr, mean ± SD)	51.73 ± 11.16	58.83 ± 8.52	0.001[b]
Tumor size (cm)			
Median	1.00 ± 0.44	1.22 ± 0.22	0.057
Range	0.6-2.5	0.4-3	
Intragastric location			
Gastric antrum	2	5	0.525
Gastric body	6	16	
Gastric fundus	30	46	
Distribution in the gastric fundus			
Proximal to cardia	24	14	< 0.001[d]
Distal to cardia	6	32	
Site within gastric wall			
Muscularis mucosae	0	1	0.449
Muscularis propria	38	66	
Sonographic characteristics			
Homogenous	36	50	0.010[d]
Hetrogenous with hyperechogenic spots	2	17	
Echogenicity in comparison with the surrounding muscle echo			
Isoechoic	38	54	0.004[b]
Hyperechoic	0	13	
Margin of the tumor			
Regular	37	61	0.212
Irregular	1	6	

[b]P < 0.01, [d]P < 0.001, leiomyoma group *vs* GIST group. GIST: Gastrointestinal tumor.

by a physician who was blinded to the pathological diagnosis 6-12 mo after ESD. Endoscopy was repeated for each patient to determine whether tumor residue or recurrence could be observed at the same time.

As mentioned above, the symptom questionnaires of the patients with GISTs and leiomyomas were investigated. During follow-up, 10 cases were excluded from the GIST group, and three from the leiomyoma group because of accompanying gastric polyps, peptic ulcers, cholecystitis, and myelocytic leukemia. Five GIST and four leiomyoma patients were lost to follow-up. After exclusion, 52 patients remained in the GIST group (24 males; age range: 30-78 years; median: 60 years). In the leiomyoma group, 31 patients were included (14 males; age range: 27-70 years; median: 53 years).

Questionnaires

The questionnaires were the Gastrointestinal Symptom Rating Scale[16] and Dyspepsia Symptom Rating Scale[17], which evaluate symptoms such as epigastric pain, heartburn, regurgitation, epigastric discomfort, nausea and vomiting, abdominal bloating, and eructation. Symptoms were assessed using a four-point scoring scale, ranging from 0-3 points based on severity with a total score of 0-21 points.

Statistical analysis

The differences in gender and EUS findings between leiomyomas and GISTs were determined. The distribution of gastrointestinal symptoms and the number of patients who recovered completely or remained unchanged were analyzed using the χ^2 test or Fisher's exact test (n < 40 or t < 1), and the patient age and tumor size were assessed using the independent t test. An independent t test or Mann-Whitney U test was performed to compare the gastrointestinal symptom scores between the GIST and leiomyoma groups. The paired t test or Wilcoxon signed-rank test was used to compare the gastrointestinal symptom scores before and after ESD in each group. Statistical analyses were performed using SPSS for Windows version 13.0 (SPSS, Chicago, IL, United States). A P-value < 0.05 was considered statistically significant.

RESULTS

EUS characteristics of gastric small GISTs

Patient and EUS characteristics of the two groups are described in Table 2. In this study, the sizes of all GISTs were < 3 cm, classifying them as gastric small GISTs. The average age in the leiomyoma group was lower than that in the GIST group (P < 0.05). Both groups were similar in terms of gender distribution (P = 0.49),

Table 3 Comparison of total gastrointestinal symptom scores of gastrointestinal tumors and leiomyomas before and after endoscopic submucosal dissection

	n	Pre-ESD	Post-ESD	*P* value
GIST	52	4.02 ± 2.54	1.34 ± 1.27	< 0.01
Leiomyoma	31	4.40 ± 2.81	2.20 ± 1.70	< 0.01
P value		0.57	0.043	

ESD: Endoscopic submucosal dissection.

intragastric location ($P = 0.525$), and originating layer within the gastric wall ($P = 0.449$). The most common site for these two tumors was the muscularis propria in the gastric fundus. As the gastric fundus is divided into proximal fundus to cardia and distal fundus based on the lowest point of the gastric fundus observed through retroflexing the tip of the scope, we found that leiomyomas were more common in the proximal fundus ($P < 0.05$). Leiomyomas tended to be smaller than GISTs ($P = 0.057$). Both groups appeared as spherical, semispherical or nodular lesions under gastroscopy. Endosonographic characteristics of leiomyomas showed homogenous hypoechoic masses and appeared as round or oval lesions with well-defined margins. However, GISTs showed heterogeneous hypoechoic masses with defined margins and hyperechoic patches ($P < 0.05$), which had higher echogenicity than the muscularis propria ($P < 0.05$).

Risk stratification of GISTs

The tumor diameters of the 67 GISTs ranged from 0.4 cm to 3 cm with an average of 1.22 ± 0.22 cm: 26 cases were ≤ 1 cm, 30 were 1-2 cm, and 11 were 2-3 cm. Postoperative histopathological results showed that all 67 GISTs were of the spindle cell type, which were occasionally accompanied by fibrosis or hyalinosis. Immunohistochemical analyses revealed that 87% were CD117 positive, 44% were CD34 positive, and 100% DOG-1 positive with mitotic counts ranging from 0 to 3 per 50 high-power fields. Based on the malignancy potential classification of GISTs, 56 cases were at very low risk and 11 were at low risk. None of these patients were given targeted drug therapy. At the end of the follow-up (6-12 mo after ESD), no tumor residue or recurrence was observed.

Comparison of total gastrointestinal symptom scores

As shown in Table 3, both total symptom scores decreased significantly after ESD ($P < 0.01$). The changes suggested that the symptoms of both groups were relieved by the procedure.

Before treatment, total symptom scores were similar in both groups ($P > 0.05$). After treatment, total symptom scores were significantly lower in the GIST group than in the leiomyoma group ($P < 0.05$), indicating that symptomatic relief by ESD was significantly greater for the GIST patients.

Gastrointestinal symptom distribution of both groups before and after ESD

Before treatment, the most common symptom of GIST patients was epigastric pain, followed by bloating and discomfort, regurgitation, heartburn, eructation, nausea, and vomiting. The duration of symptoms ranged from 1 mo to 6 years. Three (5.8%) patients were incidentally found during routine physical examinations and one was admitted for upper gastrointestinal bleeding. The most common symptom of leiomyoma patients was discomfort, followed by bloating, epigastric pain, eructation, regurgitation, heartburn, nausea, and vomiting. The duration was from 2 wk to 20 years. Two (6.5%) patients were incidentally found during routine physical examinations. Before treatment, leiomyoma patients more often had epigastric discomfort ($P = 0.021$).

After treatment, symptoms of 13 (25%) GIST patients disappeared completely. However, two (3.8%) of 52 patients with no change in total symptom scores presented with discomfort, bloating, and eructation. Five (16.1%) leiomyoma patients had complete relief of their symptoms, but there were five (16.1%) patients with unchanged total symptom scores who had discomfort and regurgitation. There were no significant differences in symptom distribution between the groups ($P > 0.05$) (Tables 4 and 5).

Comparison of symptom scores between GIST and leiomyoma patients before and after ESD

Comparison of each component of the symptom scores between GISTs and leiomyomas before and after ESD revealed no difference ($P > 0.05$) (Table 6). Each component of the symptom score demonstrated a statistically significant improvement in the GIST patients after ESD ($P < 0.05$). For leiomyoma patients, symptoms such as heartburn, nausea, vomiting, and eructation improved after treatment; however, these improvements were not statistically significant ($P > 0.05$), while other symptoms were significantly improved ($P < 0.05$).

DISCUSSION

The clinical presentations of GISTs are variable, ranging from asymptomatic to abdominal discomfort, early satiety, bloating, abdominal pain, gastrointestinal bleeding, and abdominal masses[18]. The symptoms of GISTs correlated with tumor size, site, relationship with the gastrointestinal wall, and malignancy[11,12]. Previous reports have revealed that 70% of the GIST patients presented with different levels of clinical symptoms; 20% were asymptomatic and were found incidentally during routine physical examinations or other surgeries; and 10% were found during autopsy[3]. However, small tumors are usually asymptomatic or only present with nonspecific digestive symptoms[12]. Huang *et al*[19] reported that 55.7% (59/106 cases) of the symptomatic gastric GIST patients presented with dyspepsia. Besides the case mentioned above, a female patient with refractory gastroesophageal

Table 4 Symptom distribution of gastrointestinal tumor and leiomyoma patients before and after endoscopic submucosal dissection

		Epigatric pain	Heartburn	Regurgitation	Discomfort	Nausea and vomiting	Bloating	Eructation
GIST	Pre- ESD	50% (26/52)	28.8% (15/52)	34.6% (18/52)	38.5% (20/52)	19.2% (10/52)	44.2% (23/52)	28.8% (15/52)
n = 52	Post- ESD	9.6%[a] (5/52)	11.5%[a] (6/52)	17.3%[a] (9/52)	26.9% (14/52)	3.8%[a] (2/52)	19.2%[a] (10/52)	17.3% (9/52)
Leiomyoma	Pre- ESD	38.7% (12/31)	32.2% (10/31)	32.2% (10/31)	64.5%[c] (20/31)	16.1% (5/31)	45.1% (17/31)	35.5% (11/31)
n = 31	Post- ESD	16.1% (5/31)	25.8% (8/31)	25.8% (8/31)	41.9% (13/31)	6.4% (2/31)	19.4%[a] (6/31)	16.1% (5/31)

[a]$P < 0.05$ *vs* the same group before treatment; [c]$P < 0.05$ *vs* GIST group. GIST: Gastrointestinal tumor; ESD: Endoscopic submucosal dissection.

Table 5 Comparison between complete symptom resolution and unchanged health status after endoscopic submucosal dissection

	n	Resolved completely	Unchanged
GIST	52	13	2
Leiomyoma	31	5	5
P value		0.5	0.124

GIST: Gastrointestinal tumor.

reflux disease (GERD) was reported to have a small GIST (1.4 cm × 1 cm) in the gastric fundus 6 years after GERD diagnosis. Her symptoms of regurgitation and heartburn resolved after laparoscopic surgery. O'Riain *et al*[20] described a familial GIST patient with recurrent small intestinal diverticulosis with perforation, and the authors assumed that diffuse and nodular ICC hyperplasia led to abnormal small intestinal motility, which caused decreased small intestinal peristalsis associated with small intestinal diverticulosis and perforation. These cases suggest that small GISTs may alter normal gut motility, resulting in gastrointestinal symptoms. However, there are no reports regarding the clinical symptoms of dyspepsia caused by small gastric GISTs.

Currently, it is believed that submucosal lesions are asymptomatic or produce nonspecific symptoms[21]. However, based on the data from our gastrointestinal center, most patients with submucosal lesions underwent gastroscopic examinations due to epigastric bloating, epigastric pain, regurgitation, and heartburn. Most of them were not found to have any associated gastrointestinal diseases or other diseases; therefore, the correlation between submucosal lesions and symptoms still needs to be clarified. The mechanisms underlying the symptoms are complex. The common mechanism for the symptoms of submucosal lesions may involve abnormal gastric wall distention because of the space-occupying lesion and compression of the superficial mucosa. However, different submucosal lesions may have different mechanisms for the vague and nonspecific symptoms due to their different histological origins. For example, patients with ectopic pancreatic tissue complain of abdominal pain, bloating, nausea, and vomiting. The mechanism underlying these symptoms is thought to be related to the digestive enzymes and vasoactive substances produced by the ectopic pancreas, or epigastric pain or gastrointestinal spasms resulting from the inflammation associated with gland duct obstruction of the ectopic pancreas[22].

According to the results of our study, GISTs are the most common gastric submucosal lesion, followed by leiomyomas. They are similar in size, morphology and site of origin, and are found in similar locations within the gastric wall. However, heterogeneous hypoechoic masses with hyperechoic patches, and higher echogenicity than muscularis propria are helpful for identifying gastric small GISTs. In the gastric fundus, leiomyomas are closer to the cardia, while GISTs are mainly located in the greater curvature of the fundus. The reason for this distribution is unclear. It may be related to the density of ICCs reported in the prior study[23].

Recently, the endoscopic removal of small GISTs by ESD has become technically feasible[24-26]. ESD is the most minimally invasive treatment for tumors arising from the muscularis propria; therefore, it has also the smallest effect on symptoms.

In this study, the fact that only 5.8% of GIST patients and 6.5% of leiomyoma patients were found incidentally during routine physical examinations suggests that most of these patients presented with gastrointestinal symptoms. Total symptom scores of both groups decreased significantly after ESD, suggesting that the symptoms were probably caused by the submucosal lesions. After ESD, total symptom scores were significantly lower in the GIST group than in the leiomyoma group, and each component of the symptom score demonstrated a statistically significant improvement in the GIST patients after ESD ($P < 0.05$), including epigastric pain, heartburn, regurgitation, epigastric discomfort, nausea and vomiting, abdominal bloating, and eructation. For leiomyoma patients, symptoms such as heartburn, nausea, vomiting, and eructation improved after treatment; however, these improvements were not statistically significant ($P > 0.05$). These data suggest that GISTs may have more impact on the symptoms.

GISTs are thought to originate from ICCs or their precursors because they share similarities such as cellular ultrastructure[27]. High levels of CD117, CD34, protein kinase Cθ, and nestin are expressed in both GISTs and ICC cells[8,28-30]. Diffuse ICC hyperplasia has been described with c-kit mutation, and is associated with the development of multiple GISTs[31,32]. In addition, some expressed genes of GISTs are related to the electrophysiological activity of ICCs, such as the potassium ion channel gene *KCNK3*, *KCNK2*[33], and the calcium-activated

Table 6 Comparison of symptom scores between gastrointestinal tumors and leiomyomas before and after endoscopic submucosal dissection

		Epigatric pain	Heartburn	Regurgitation	Discomfort	Nausea and vomiting	Bloating	Eructation
GIST	Pre ESD	0.80 ± 0.90	0.63 ± 1.08	0.55 ± 0.87	0.70 ± 0.98	0.27 ± 0.62	0.70 ± 0.90	0.36 ± 0.61
n = 52	Post ESD	0.13 ± 0.46[a]	0.13 ± 0.41[a]	0.22 ± 0.57[a]	0.32 ± 0.47[a]	0.05 ± 0.21[a]	0.27 ± 0.49[a]	0.21 ± 0.46[a]
Leiomyoma	Pre ESD	0.64 ± 0.86	0.60 ± 0.82	0.60 ± 0.82	1.00 ± 0.82	0.20 ± 0.50	1.00 ± 0.82	0.56 ± 0.92
n = 31	Post ESD	0.24 ± 0.52[a]	0.32 ± 0.56	0.32 ± 0.56[a]	0.52 ± 0.65[a]	0.08 ± 0.28	0.40 ± 0.64[a]	0.32 ± 0.69

[a]$P < 0.05$ *vs* before treatment. ESD: Endoscopic submucosal dissection.

chloride channel gene *DOG-1*[34-36]. Furuzono *et al*[37] have found that malignant GIST cells preserve several, but not all, ionic mechanisms underlying pacemaker activity in ICCs. However, leiomyomas originate from smooth muscle cells. Based on these data, we suggest that GISTs, especially small GISTs, likely retain partial biological features of ICCs and somehow induce or aggravate abnormal gastrointestinal electric activity, resulting in gastrointestinal dyspepsia symptoms. This mechanism differs from that of leiomyomas.

In addition, the symptoms of only 25% of GIST patients and 16% of leiomyoma patients completely resolved after ESD. The incomplete resolution of symptoms for the other patients suggests that the mechanism for the gastrointestinal symptoms is complicated and may relate to the formation of postoperative scar, visceral hypersensitivity, and psychological factors.

The symptoms of small gastric GISTs may mimic those of functional dyspepsia. Small gastric GISTs may have more impact on symptoms than gastric leiomyomas, due to their different histological origins. The incidence of gastric micro-GISTs is higher than that of gastric clinical GISTs. We suggest that some symptoms in patients with functional dyspepsia are the result of functional alteration of gut peristalsis due to the increased number of ICCs in a slow-growing GIST, even when the tumor is small and difficult to detect by routine investigations. Thus, an alternative diagnosis should be considered when treating patients with functional dyspepsia who fail to respond for a prolonged period. However, multicenter studies with a larger cohort of patients, and more research about gastric myoelectrical activity and the physiology of gut motility related to GISTs are needed.

COMMENTS

Background

Gastrointestinal stromal tumors (GISTs) are among the most common mesenchymal tumors of the gastrointestinal tract, and are thought to originate from the interstitial cells of Cajal (ICCs). These cells are involved in the regulation of gastrointestinal motility, and the ionic mechanism underlying their pacemaker activity is preserved by GISTs. Therefore, these tumors, particularly when small, may preserve the biological functions of these cells, thereby disturbing the normal gastric myoelectrical activity and resulting in dyspepsia symptoms. The aim of the present study was to explore whether gastric small GISTs - the neoplastic transformation of the ICCs - result in gastrointestinal dyspepsia symptoms.

Research frontiers

GISTs are thought to originate from ICCs or their precursors because they share similarities such as cellular ultrastructure. Some expressed genes of GISTs are related to the electrophysiological activity of ICCs. Furuzono *et al* have found that malignant GIST cells preserve several, but not all, ionic mechanisms underlying pacemaker activity in ICC.

Innovations and breakthroughs

Several case reports have suggested that GISTs which may conserve the function of ICCs could alter the motility of the gastrointestinal tract, especially when the tumor is sufficiently small. The symptoms may mimic those of functional gastrointestinal disorders. However, there are no reports regarding the clinical symptoms of dyspepsia caused by small gastric GISTs.

Applications

The symptoms of some small gastric GISTs may mimic those of functional dyspepsia. The incidence of gastric micro-GISTs is higher than that of gastric clinical GISTs. The authors suggested that some functional dyspepsia symptoms result from functional alteration of gut peristalsis due to the increased number of ICCs in a slow-growing GIST, even when the tumor is significantly small and thus difficult to detect by routine investigation. Therefore, an alternative diagnosis should be considered when treating patients with functional dyspepsia who fail to respond for a prolonged period.

Terminology

GISTs are mesenchymal tumors derived from the ICCs. The incidence of GISTs is 10-20 cases per million and the stomach is the most common location (60%-70%). ICCs are specialized cells in the gastrointestinal tract that generate the rhythmic electrical and contractile activity that exists from the stomach to the rectum, and mediate enteric motor neurotransmission.

Peer review

The manuscript submitted by Yu *et al* addresses an important topic related to GISTs and the symptom severity following removal of the tumor. In general, the manuscript is well written. There are some minor sentence structure issues that should be resolved by the authors when reading through the manuscript. The statistics are sound and the overall methods section is well written.

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RETROSPECTIVE STUDY

Impact of medical therapy on patients with Crohn's disease requiring surgical resection

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Abstract

AIM: To evaluate the impact of medical therapy on Crohn's disease patients undergoing their first surgical resection.

METHODS: We retrospectively evaluated all patients with Crohn's disease undergoing their first surgical resection between years 1995 to 2000 and 2005 to 2010 at a tertiary academic hospital (St. Paul's Hospital, Vancouver, Canada). Patients were identified from hospital administrative database using the International Classification of Diseases 9 codes. Patients' hospital and available outpatient clinic records were independently reviewed and pertinent data were extracted. We explored relationships among time from disease diagnosis to surgery, patient phenotypes, medication usage, length of small bowel resected, surgical complications, and duration of hospital stay.

RESULTS: Total of 199 patients were included; 85 from years 1995 to 2000 (cohort A) and 114 from years 2005 to 2010 (cohort B). Compared to cohort A, cohort B had more patients on immunomodulators (cohort A *vs* cohort B: 21.4% *vs* 56.1%, $P < 0.0001$) and less patients on 5-aminosalysilic acid (53.6% *vs* 29.8%, $P = 0.001$). There was a shift from inflammatory to stricturing and penetrating phenotypes (B1/B2/B3 38.8% *vs* 12.3%, 31.8% *vs* 45.6%, 29.4% *vs* 42.1%, $P < 0.0001$). Both groups had similar median time to surgery. Within cohort B, 38 patients (33.3%) received anti-tumor necrosis factor (TNF) agent. No patient in cohort A was exposed to anti-TNF agent. Compared to patients not on anti-TNF agent, ones exposed were younger at diagnosis (anti-TNF *vs* without anti-TNF: A1/A2/A3 39.5% *vs* 11.8%, 50% *vs* 73.7%, 10.5% *vs* 14.5%, $P = 0.003$) and had longer median time to surgery (90 mo *vs* 48 mo, $P = 0.02$). Combination therapy further extended median time to surgery. Using time-dependent multivariate Cox proportional hazard model, patients who were treated with anti-TNF agents had a significantly higher risk to surgery (adjusted hazard ratio 3.57, 95%CI: 1.98-6.44, $P < 0.0001$) compared to those without while controlling for gender, disease phenotype, smoking status, and immunomodulator use.

CONCLUSION: Significant changes in patient phenotypes and medication exposures were observed between the two surgical cohorts separated by a decade.

Key words: Crohn's disease; Surgery; Medication; Phenotype; Biologics; Anti-tumor necrosis factor; Immunomodulators; Inflammatory bowel disease

Core tip: Comparing two cohorts separated by a decade of Crohn's disease patients who required surgical resections, this study showed significant changes in patient phenotypes and medication usage. Those that

required surgery shifted from more inflammatory to stricturing and penetrating phenotypes, and had more immunomodulators but less 5-aminosalysilic acid exposures. Patients treated with biologics had significantly longer time from Crohn's disease diagnosis to surgery. However, they were at increased risk for surgery, suggesting that biologics were often used too late in the patients' treatment courses.

Fu YTN, Hong T, Round A, Bressler B. Impact of medical therapy on patients with Crohn's disease requiring surgical resection. *World J Gastroenterol* 2014; 20(33): 11808-11814 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i33/11808.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i33.11808

INTRODUCTION

Crohn's disease (CD) is a chronic idiopathic inflammatory condition affecting the gastrointestinal tract. It often takes a relapsing and remitting course. Despite advances in medical management, many patients eventually develop complications requiring surgical interventions[1-3]. Up to 60% of CD patients require surgery within ten years of their disease diagnosis[3,4]. Although surgical resection can be associated with long-lasting remission, it has many inherent complications[5]. Therefore, it is usually reserved for cases where medical management fails. To avoid or delay surgery while keeping patients in remission is a desirable goal in CD management.

Medical management of CD traditionally involved a step-up approach[6,7]. Once in remission, patients are maintained with an immunomodulator (IM) or anti-tumor necrosis factor (TNF) agent. Infliximab was approved by Health Canada for use in CD treatment in 2001 and adalimumab was approved in 2008. They have demonstrated efficacy in inducing and maintaining CD remission in patients who have previously failed conventional therapy[8-13]. However, due to costs and reimbursement restrictions, early use of anti-TNF agents is limited in Canada. The usual treatment approach remains in a step-up fashion. This study evaluated the impact of medical treatments on CD patients undergoing their first surgical resection at a tertiary academic hospital. In particular, we assessed time from disease diagnosis to their first surgical resection.

MATERIALS AND METHODS

We retrospectively evaluated all patients with Crohn's disease who had their first surgical resection in years 1995 to 2000 (cohort A) and years 2005 to 2010 (cohort B) at a tertiary academic hospital (St. Paul's Hospital, Vancouver, Canada). These two time cohorts were selected based on the availability of anti-TNF agents in Canada. We intended to further delineate the impact of anti-TNF agents on surgical resections. Only patients with excisional intestinal surgery were included in this study. Stricturoplasty, bypass, and elective surgical treatment of abscess were not considered. The surgical approach over the study time period did not change at this hospital. Patients were initially identified using an electronic search of the hospital's medical records using the International Classification of Diseases 9 codes. Trained abstractors (Fu YTN, Hong T, Round A) conducted standardized chart reviews on all patients. Both hospital and available outpatient clinic charts were reviewed. The diagnosis of Crohn's disease was accepted if standard radiographic, endoscopic, or histological criteria were documented in the medical record. All diagnosis was confirmed with surgical pathology specimens. Crohn's disease phenotypes at the time of surgery were recorded. Pertinent information regarding the patients' baseline demographics, disease phenotypes, medication exposure, time from disease diagnosis to surgery, and details surrounding the first surgery specifically amount of small intestine resected, post-operative complications and length of hospital stay were retrieved from their charts. Azathioprine, 6-mercaptopurine and methotrexate were designated as IM. The Montreal classification was used to denote disease phenotypes[14]. This study received full institutional ethics approval.

Statistical analysis

We performed χ^2 or Fisher's exact tests for categorical variables and *t*-tests or Wilcoxon rank-sum test for continuous variables. Kruskal-Wallis test was used to compare the time from disease diagnosis to surgery among three patient groups; patients in the 1995-2000 cohort (cohort A), patients exposed to, and not exposed to anti-TNF agent in the 2005-2010 cohort (cohort B). Time-dependent adjusted multivariate Cox proportional hazard model was used to assess risk to surgical resection. We performed all statistical analyses using SAS Version 9.2 (SAS Institute, NC, United States). All statistical tests were two-sided with a 0.05 significance level.

RESULTS

Eighty five patients with Crohn's disease had their first surgical resection between the years 1995 to 2000 (cohort A) and 114 patients had first surgery between years 2005 to 2010 (cohort B).

Comparing the two time cohorts, the patients had similar median age at the time of surgery but there were more males in 2005-2010 (cohort B) (Table 1). Significantly different disease phenotypes were identified. There was a shift from inflammatory to stricturing and penetrating diseases as well as a change from ileal and colonic to ileocolonic diseases (Table 1; cohort A *vs* cohort B: B1/B2/B3 38.8%/31.8%/29.4% *vs* 12.3%/45.6%/42.1%, $P < 0.0001$; L1/L2/L3 32.9%/28.2%/38.8% *vs* 26.3%/14.9%/58.8%, $P = 0.01$). The patients also had significantly different medication

Table 1 Demographic and surgical details for Crohn's disease patients with first resections in years 1995-2000 (cohort A) and years 2005-2010 (cohort B)

	Cohort A 1995-2000 (n = 85)	Cohort B 2005-2010 (n = 114)	P value
Age	33 (± 12.1)	31.5 (±13.9)	0.440
Gender (M/F)	30.6 (26)/69.4 (59)	54.4 (62)/ 45.6 (52)	0.001
Montreal classification			
Age at diagnosis (A1/A2/A3)	9.8%/74.4%/15.9%	21.1%/65.8%/13.2%	0.110
Disease behavior (B1/B2/B3)	38.8%/31.8%/29.4%	12.3%/45.6%/42.1%	< 0.0001
Disease location (L1/L2/L3)	32.9%/28.2%/38.8%	26.3%/14.9%/58.8%	0.010
Medication exposure			
5-ASA	53.6 (45)	29.8 (34)	0.001
CS	69.1 (58)	75.4 (86)	0.320
IM	21.4 (18)	56.1 (64)	< 0.0001
Surgical details			
Time from diagnosis to surgery (mo)	72 ± 83.8	72 ± 89.5	0.710
Amount of small bowel resected (cm)	21 ± 12.6	23 ± 17.5	0.820
Length of hospital stay (d)	10 ± 16.7	9 ± 8.0	0.050

Data are expressed as absolute numbers (percentage) or mean ± SD. 5-ASA: 5-aminosalysilic acid; CS: Corticosteroid; IM: Immunomodulator; M: Male; F: Female.

Table 2 Demographic and Surgical details for patients treated with and without anti-tumor necrosis factoragents in years 2005-2010 (cohort B)

	Anti-TNF (n = 38)	Without Anti-TNF (n = 76)	P value
Age	29.5 (± 13.14)	33.5 (± 14.05)	0.10
Gender (M/F)	60.5 (23)/39.5 (15)	51.3 (39)/48.7 (37)	0.35
Smoking status (yes)	26.3 (10)	23.7 (18)	0.82
Montreal classification			
Age at diagnosis (A1/A2/A3)	39.5%/50%/10.5%	11.8%/73.7%/14.5%	0.003
Disease behavior (B1/B2/B3)	18.4%/39.5%/42.1%	9.2%/48.7%/42.1%	0.33
Disease location (L1/L2/L3)	23.7%/29%/47.4%	27.6%/7.9%/64.5%	0.01
Medication exposure			
5-ASA	29 (11)	30.3 (23)	0.88
CS	81.6 (31)	72.4 (55)	0.28
IM	79 (30)	44.7 (34)	0.001
Surgical details			
Time from diagnosis to surgery (mo)	90 ± 63.1	48 ± 100.2	0.02
Amount of small bowel resected (cm)	23 ± 12.4	21.5 ± 19.3	0.92
Length of hospital stay (d)	9 ± 8.6	10 ± 7.7	0.76

Data are expressed as absolute numbers (percentage) or mean ± SD. 5-ASA: 5-aminosalysilic acid; CS: Corticosteroid; IM: Immunomodulator; M: Male; F: Female.

exposure. The later cohort B had significantly less 5-aminosalysilic acid (5-ASA) but more IM exposure (Table 1; cohort A *vs* cohort B: 5-ASA 53.6% *vs* 29.8%, $P = 0.01$; IM 21.4% *vs* 56.1%, $P < 0.0001$). There was no difference in corticosteroid exposure, surgical details, median length of hospital stay, post-operative complication rate, and median time from disease diagnosis to first surgical resection. The median time from disease diagnosis to surgery was 72 mo.

Within the 2005-2010 cohort (cohort B), 38 patients (33.3%) received anti-TNF therapy; 18 treated with infliximab, five with adalimumab, and 15 with both agents sequentially. Only eight subjects were treated with anti-TNF agent alone, and all others were treated concomitantly with IM. No patient was exposed to anti-TNF in the earlier cohort A. Patients treated with and without anti-TNF agent had comparable median age at the time of surgery and gender distribution (Table 2). However, those received anti-TNF agent were younger at disease diagnosis (Table 2; anti-TNF *vs* without anti-TNF; 39.5% *vs* 11.8%, 50% *vs* 73.7%, 10.5% *vs* 14.5%, $P = 0.003$), had more colonic diseases (23.7% *vs* 27.6%, 29% *vs* 7.9%, 47.4% *vs* 64.5%, $P = 0.01$), and higher IM usage (79% *vs* 44.7%, $P = 0.001$). No difference was seen in corticosteroid and 5-ASA exposure, median length of hospital stay, and post-operative complication rate.

Patients who received anti-TNF agent had longer median time from disease diagnosis to first surgical resection (Figure 1; 90 mo *vs* 48 mo, $P = 0.02$). Combination therapy with anti-TNF and IM lead to much extended median time to surgery when compared to anti-TNF or IM alone (Figure 2; 96 mo *vs* 60 mo, 96 mo *vs* 54 mo, $P = 0.03$). There was no difference in smoking status ($P = 0.82$) or disease behavior ($P = 0.33$) in ones treated with and without anti-TNF agent (Table 2). The median time to surgery for patients not receiving anti-TNF therapy in

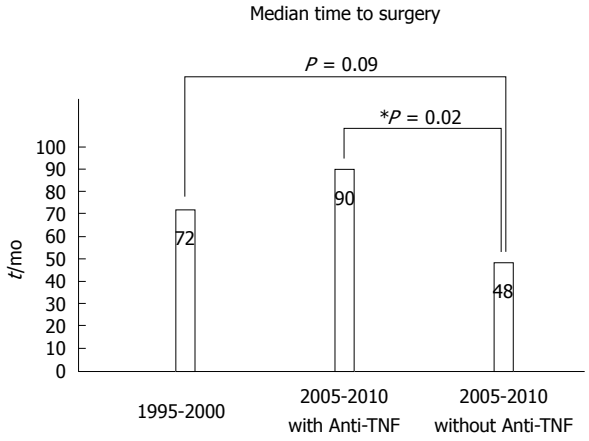

Figure 1 Patients treated with anti-tumor necrosis factor agents had significantly longer median time from Crohn's disease diagnosis to surgery. TNF: Tumor necrosis factor.

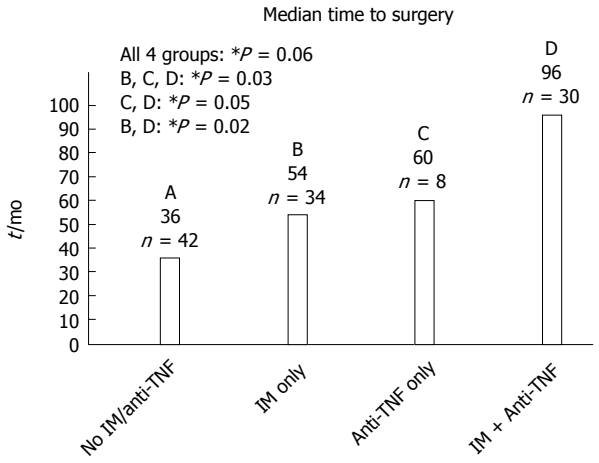

Figure 2 Combination therapy using immunomodulator and anti-tumor necrosis factor agent further extended the median time from Crohn's disease diagnosis to surgery for patients requiring surgery in years 2005 to 2010 (cohort B). TNF: Tumor necrosis factor; IM: Immunomodulator.

the 2005-2010 cohort (cohort B) was shorter compared to those in the 1995-2000 cohort (cohort A) (Figure 1; 48 mo *vs* 72 mo).

Risk for surgical resection for patients in the 2005-2010 cohort (cohort B) was assessed using time-dependent multivariate Cox proportional hazard model controlling for gender, disease phenotype, smoking status, IM, and anti-TNF use. Patients who were treated with anti-TNF agent had a significantly higher time-dependent risk to surgery (adjusted hazard ratio (HR) = 3.57, 95%CI: 1.98-6.44, $P < 0.0001$) compared to those without. The median time from disease diagnosis to administration of anti-TNF agent was 66 mo and the median time from administration of anti-TNF agent to surgery was 14 mo. This suggests that anti-TNF agent was used too late in the treatment course.

DISCUSSION

The recent shift in medical management of Crohn's disease has decreased the cumulative surgical rate of CD patients[15-20]. Our study echoed the observed trends that more IM but less 5-ASA products are currently used in CD management[18-22]. Immunomodulators are effective maintenance medications and azathioprine has been shown to modestly lower the risk of surgery[23-25]. A recent meta-analysis showed a combined pooled HR of 0.59 for first intestinal resection with thiopurine use[26]. Therefore, selecting patients who are most likely to benefit from IM may change the surgical rate of patients with CD.

Anti-TNF agents are very efficacious in treating refractory luminal and fistulising CD[8-13]. However, once CD is complicated with strictures and stenosis, surgical resection is typically the best therapeutic option. Our study confirmed that anti-TNF agents are the best for treating inflammatory CD[3,19,20,25,27]. We observed a significant shift in disease phenotypes from inflammatory to stricturing and penetrating phenotypes in patients requiring surgical resection. This shift in phenotypes suggests that the changes in medical management have lead to more success in managing patients with inflammatory CD, but the therapies are less effective in structuring and penetrating diseases. Additionally, our study infers that younger patients had more aggressive disease and they were more likely to require anti-TNF agents as rescue treatment.

The reported time from CD diagnosis to surgery varies widely in literature, ranging from one to 19 mo[17,27,28]. Our two cohorts had the same median time to surgery of 72 mo, much longer than reported in literature. Such discrepancies may be due to differences in regional surgical referral pattern. Interestingly, patients who did not receive anti-TNF therapy in our later cohort B (2005-2010) had shorter time to surgery than those in the earlier cohort A (1995-2000) (48 mo *vs* 72 mo). This suggests that either patients in the later cohort had more aggressive disease, or the phenotypes of the patients being seen in this tertiary centre were more prone to surgery. Stricturing and penetrating phenotypes were more common in the later cohort which is the likely cause of the shorter time to resection. Patients with disease status that may warrant surgical resection in the earlier decade were, instead, being placed on more aggressive medical therapy such as anti-TNF agent. Additionally, surgeons in the later time cohort may be more selective in operating on only the sicker patients although the overall surgical approach to CD management did no change throughout the study period.

A recent meta-analysis showed infliximab reduces hospitalization and major surgery[29]. Our study found that anti-TNF agents and combination therapy extend

time from CD disease diagnosis to first surgical resection in CD patients. However, patients who received anti-TNF agents in the later cohort had a higher time-dependent risk to resective surgery. This suggests that anti-TNF agent was used too late in the patient's treatment course at our center. With earlier introduction of anti-TNF agent, the patients may have a different time course to surgery. In Bouguen and Peyrin-Biroulet's review, the surgical risk for adult CD within 5 year at a referral centre ranged from 17% to 35% in pre-anti-TNF era and 18% to 33% in anti-TNF era[4]. Our study reflects the changes described that, with wider and earlier use of IM and anti-TNF agents, we begin to see how these medications can alter the natural history of CD[4].

There are limitations to our study. As this is a single-centered, retrospective study with a relatively small sample size, we are unable to establish causal relationships. Although there were only 38 patients who received anti-TNF therapy in this study, it is proportionally higher when compared to the literature[18,20,21,30,31]. We found no significant difference in confounding variables to surgery such as smoking status and disease behavior in anti-TNF exposed and unexposed patients. A significantly higher proportion of patients were diagnosed at a young age in the anti-TNF exposed group. Younger patients with CD often have more aggressive disease that may lead to early surgery and requirement of anti-TNF agents[32-35]. Despite this, anti-TNF exposed still demonstrated longer median time to surgical resection. Studies conducted in tertiary centres may have skewed patient populations with more aggressive phenotypes as our study demonstrated.

Significant changes in patient phenotypes and medication exposure were observed between the two surgical cohorts separated by a decade. Patients received ant-TNF agents had prolonged time to surgery and those on combination therapy had the longest median time to their first surgical resection. However, the study result suggests that anti-TNF agent was often used too late in patient's treatment course.

COMMENTS

Background

Since the past decade, new medical therapies are now available for treatment of Crohn's disease (CD). Majority of CD patients require surgery within ten years of their disease diagnosis. To avoid or delay surgery while keeping patients in remission is a desirable goal in CD management.

Research frontiers

Immunomodulators are effective maintenance medications for CD. Anti-tumor necrosis factor (TNF) agents have demonstrated efficacy in inducing and maintaining CD remission in patients who have previously failed conventional therapy. However, the impact of medication on the natural progression of CD is unclear. This study evaluated changes in patient phenotypes, medication exposures, and time from disease diagnosis to surgery in CD patients requiring surgical resection.

Innovations and breakthroughs

Patients with CD requiring surgical resections shifted from more inflammatory to stricturing and penetrating phenotypes, and had more immunomodulators but less 5-aminosalysilic acid exposures. Patients treated with combination therapy had the longest time from disease diagnosis to their first surgical resection. Biologics were likely used too late in the patients' treatment course.

Applications

Early and appropriate use of medical therapy may alter the natural progression of Crohn's disease by preventing or delaying surgical resection. Combination therapy may lead to extended time from disease diagnosis to surgery.

Terminology

Immunomodulators include azathioprine, 6-mercaptopurine and methotrexate. Anti-TNF agents or biologics include infliximab and adalimumab. Combination therapy implies simultaneous use of an immunomodulator and an anti-TNF agent. Montreal classification denotes Crohn's disease phenotypes based on age of diagnosis (A1 age < 16, A2 age 17-40, A3 age > 40), disease behavior (B1 non-stricturing/non-penetrating, B2 stricturing, B3 penetrating), and disease location (L1 ileal, L2 colonic, L3 ileocolonic). Modifiers for upper gastrointestinal (L4) and/or perianal (p) disease involvement can be applied.

Peer review

The paper focuses on a very interesting issue and reports data from a reasonably large series of patients. It would be nice to know about patients in the middle (years 2000-2005) in which a mixed population is present and a comparison between the beginning of anti-TNF use and a more mature utilization could be performed.

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RETROSPECTIVE STUDY

Non invasive blood flow measurement in cerebellum detects minimal hepatic encephalopathy earlier than psychometric tests

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Abstract

AIM: To assess whether non invasive blood flow measurement by arterial spin labeling in several brain regions detects minimal hepatic encephalopathy.

METHODS: Blood flow (BF) was analyzed by arterial spin labeling (ASL) in different brain areas of 14 controls, 24 cirrhotic patients without and 16 cirrhotic patients with minimal hepatic encephalopathy (MHE). Images were collected using a 3 Tesla MR scanner (Achieva 3T-TX, Philips, Netherlands). Pulsed ASL was performed. Patients showing MHE were detected using the battery Psychometric Hepatic Encephalopathy Score (PHES) consisting of five tests. Different cognitive and motor functions were also assessed: alterations in selective attention were evaluated using the Stroop test. Patients and controls also performed visuo-motor and bimanual coordination tests. Several biochemical parameters were measured: serum pro-inflammatory interleukins (IL-6 and IL-18), 3-nitrotyrosine, cGMP and nitrates+nitrites in plasma, and blood ammonia. Bivariate correlations were evaluated.

RESULTS: In patients with MHE, BF was increased in cerebellar hemisphere (P = 0.03) and vermis (P = 0.012) and reduced in occipital lobe (P = 0.017). BF in cerebellar hemisphere was also increased in patients without MHE (P = 0.02). Bimanual coordination was impaired in patients without MHE (P = 0.05) and much more in patients with MHE (P < 0.0001). Visuo-motor coordination was impaired only in patients with MHE (P < 0.0001). Attention was slightly affected in patients without MHE and more strongly in patients with MHE (P

< 0.0001). BF in cerebellar hemisphere and vermis correlated with performance in most tests of PHES [(number connection tests A (NCT-A), B (NCT-B)and line tracing test] and in the congruent task of Stroop test. BF in frontal lobe correlated with NCT-A. Performance in bimanual and visuomotor coordination tests correlated only with BF in cerebellar hemisphere. BF in occipital lobe correlates with performance in the PHES battery and with CFF. BF in cerebellar hemisphere correlates with plasma cGMP and nitric oxide (NO) metabolites. BF in vermis cerebellar also correlates with NO metabolites and with 3-nitrotyrosine. IL-18 in plasma correlates with BF in thalamus and occipital lobe.

CONCLUSION: Non invasive BF determination in cerebellum using ASL may detect MHE earlier than the PHES. Altered NO-cGMP pathway seems to be associated to altered BF in cerebellum.

Key words: Arterial spin labelling; Neurological impairment; Blood flow; Cerebellum; Minimal hepatic encephalopathy

Core tip: Patients with minimal hepatic encephalopathy (MHE) show neurological impairment in specific tasks to which selective regional alterations in blood flow (BF) could contribute. Arterial spin labeling (ASL), a non-invasive magnetic resonance technique, quantitatively measures cerebral perfusion. We analyzed BF by ASL in different brain areas of controls and cirrhotic patients without and with MHE. We found that BF is more affected in cerebellum than in other areas of cirrhotic patients and that BF determination in cerebellum using ASL may detect MHE earlier than the Psychometric Hepatic Encephalopathy Score battery. Altered nitric oxide-cGMP pathway seems to be associated to altered BF in cerebellum.

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INTRODUCTION

Patients with minimal hepatic encephalopathy (MHE) show selective alterations in specific functions and tasks such as visuo-spatial orientation and perception, complex tasks requiring attention and motor abilities[1-5]. This suggests that the brain areas modulating these tasks are more affected than others in patients with liver disease and MHE. The reasons for this "region selective" sensitivity may include selective alterations in blood flow (BF)[6].

Invasive positron emission tomography (PET) and single photon emission computed tomography (SPECT) techniques have shown altered cerebral blood flow (CBF) patterns in cirrhotic patients, but there is a large variability between the results published[6-9]. Some studies show redistribution of the BF from cortex to thalamus and cerebellum[6]. However, Iwasa *et al*[7,8] reported reduced BF both in cortex, cerebellum and cingulate gyrus of cirrhotic patients. These differences may reflect different grades in the progression of the disease.

The possible contribution of alterations in BF in specific brain areas to neurological alterations in patients with MHE remains unclear. Ahl *et al*[6] found that there was no association between changes in CBF and neuropsychiatric status. However, Catafau *et al*[9] reported a correlation between altered BF in basal ganglia and reduced performance in the "Luria Motor Alternances" and "Purdue Pegboard" tests. Iwasa *et al*[7,8] did not find correlations between reduced BF in frontal lobe and neuropsychological alterations, but later reported a correlation between reduced BF in cingulate gyrus and performance in psychometric tests. This suggests that BF alterations in some specific regions could be involved in the mechanisms leading to certain cognitive and motor alterations.

The above studies were performed using invasive techniques. It would be useful to have non-invasive procedures to analyze regional CBF and evaluate if BF in some region is useful to detect MHE and/or specific neurological alterations. This would allow early treatment and follow-up of the eficiency of therapeutic treatments.

Arterial spin labeling (ASL) is a completely non-invasive magnetic resonance technique that quantitatively measures cerebral perfusion by magnetically labeling protons in arterial blood water. The labeled protons travel through the vascular tree and exchange water with non labeled cerebral tissue. The perfusion cerebral image is obtained by substracting the images with the labeled and unlabeled protons[10].

An important modulator of CBF is the nitric oxide (NO)-cGMP system[11], which also modulates some forms of learning and memory. Altered function of the glutamate-NO-cGMP pathway in brain is responsible for some types of cognitive impairment in animal models of MHE. Normalizing this system by pharmacological treatments restores learning ability[12-14]. It is therefore likely that altered NO-cGMP system could contribute to alter BF in some brain areas which could contribute to cognitive impairment. Detection of altered CBF by magnetic resonance could serve as an indicator of mild cognitive impairment in MHE. Identifying some parameter in blood reflecting altered CBF in patients with liver disease would be also useful.

The aims of this work were to: (1) analyze by ASL the BF in brain areas of patients with liver cirrhosis; (2) assess whether patients with or without MHE show similar or different alterations; (3) assess if BF in some brain area may be a good indicator of MHE or of specific neu-

Table 1 Composition of the groups and clinical data (mean ± SD)

	Range	Control	Patients without MHE	Patients with MHE
Total individuals		14	24	16
Age		55 ± 9	58 ± 6	65 ± 9
Alcohol			24	16
Ascitis			2	5
Child Pugh A/B/C			21/3/0	11/3/2
MELD			8.9 ± 3.5	9.5 ± 3.4
AST (mU/mL)	1-37	20 ± 4.0	73 ± 56[b]	82.5 ± 58[b]
ALT (mU/mL)	1-41	18 ± 6.0	77 ± 24[b]	90.1 ± 24[b]
GGT (mU/mL)	10-49	26.7 ± 5.0	86.4 ± 60[b]	106 ± 64[b]
Uric acid (mg/dL)	2.5-7	4.0 ± 1.0	6.2 ± 2.0	5.73 ± 2.3
Creatinine (mg/dL)	0.5-1.3	0.92 ± 0.1	1.1 ± 0.2	1.2 ± 0.2
Cholesterol (mg/dL)	140-200	172 ± 22	175 ± 44	167 ± 55
Triglycerides (mg/dL)	40-160	95 ± 32	111 ± 64	119 ± 64
Bilirubin (mg/dL)	0.1-1	0.6 ± 0.2	1.7 ± 0.7[b]	2.3 ± 0.6[b]
Albumin (g/dL)	3.5-5	4.4 ± 0.2	3.7 ± 0.6[b]	2.9 ± 0.6[b,c]
Prothrombin time (s)		13 ± 1.3	24 ± 4[b]	30 ± 4b
Fibrinogen (g/L)	2-4	3.1 ± 1.0	3.3 ± 1.3	3.6 ± 1.2
Alkaline phosphatase (mU/mL)	50-250	147 ± 53	216 ± 77[b]	314 ± 96[b,c]
Erythrocytes	4.2-6.1	4.6 ± 0.4	4.3 ± 0.7	3.4 ± 0.6
Leucocytes	4.8-10.8	6.5 ± 1.3	6 ± 2.6	5.5 ± 2.0
Neutrophils (%)	55-75	55 ± 7.4	54 ± 6.2	59 ± 9.3
Lymphocytes (%)	17-45	35 ± 6.0	29 ± 10	27 ± 9.4
Monocytes (%)	2-8	6.0 ± 1.3	8.4 ± 3.0[b]	10 ± 2.6[b]
Eosinophils (%)	1-4	3.3 ± 2.0	2.4 ± 1.2	1.7 ± 1.0
Basophils (%)	0.05-0.5	0.5 ± 0.2	0.6 ± 0.3	0.6 ± 0.1

[b]$P < 0.01$ *vs* control, [c]$P < 0.05$ *vs* without minimal hepatic encephalopathy (MHE). MELD: Model for end stage liver disease. Values that are significantly different from controls are indicated by superscripts.

rological alterations; and (4) assess whether some peripheral parameter correlates with BF. As possible peripheral indicators we determined parameters of the NO-cGMP system in blood. We also measured ammonia levels, parameters related with inflammation and 3-nitrotyrosine[15].

MATERIALS AND METHODS

Patients and controls

Forty patients with liver disease and 14 controls were enrolled after written informed consent. Inclusion criteria: patients were recruited from the outpatient clinics at Hospitals Clinico Universitario and Arnau de Vilanova, in Valencia and were included if they had clinical, biochemical and histological evidence of liver cirrhosis. For controls liver disease was discarded by clinical, analytical and serologic analysis. Patients were excluded if they had evidence of overt hepatic encephalopathy (HE) by the West Haven criteria, decompensate diabetes, renal dysfunction, hyponatremia, neurological disease, cardiovascular disease or antibiotic use. Patients had to be abstinent from alcohol for six months. Patients were not on any specific therapy for HE. After a standard history and physical examination, blood was drawn for biochemical measurements.

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethical Committee of the Hospital. After performing the psychometric tests, patients were classified as without or with MHE (see below). The study includes therefore three groups: (1) controls; (2) patients without MHE; and (3) patients with MHE. MRI examination was performed one week after the psychometric tests. The composition of groups, age, analytical data and aetiology of the disease are in Table 1.

Assessment of neuropsychometric status

Diagnosis of MHE: Patients showing MHE were detected using the battery Psychometric Hepatic Encephalopathy Score (PHES). Classification of patients as "with" or "without" MHE was based only on PHES performance, considered as the "gold standard"[16]. PHES comprises 5 psychometric tests: digit symbol test (DST) evaluates processing speed and working memory, number connection tests A (NCT-A) and B (NCT-B) mental processing speed and attention; serial dotting test (SD) and line tracing test (LTT) visuo-spatial coordination. The scores were adjusted for age and education level using Spanish normality tables (www.redeh.org). Patients were classified as having MHE when the score was less than −4 points[17].

Evaluation of attention with the Stroop test: The Stroop task evaluates selective attention. We used a colour-word version of the Stroop task[18]. Each individual performed sequentially the congruent, neutral and incongruent tasks, 45 s per task. The number of items correctly named was quantified and adjusted by age[19].

Visuo-motor coordination: This task was performed as described in reference[20].

Bimanual coordination: This task was performed as described in reference[21].

Critical flicker frequency: The CFF was measured as in reference references[22,23].

Obtention of plasma and serum

Venous blood (5 mL) was taken in BD Vacutainer tubes with or without EDTA (plasma and serum, respectively) and centrifuged at 500 *g* for 10 min. The supernatant was collected, and stored frozen at -80 ℃.

Biochemical determinations in blood

Nitrates + nitrites in plasma were measured as previously[24]. Serum IL-6 and IL-18 were determined using ELISA kits (Pierce Biotechnology, United States) and Bender MedSystems GmbH (Vienna, Austria), respectively. Plasma cGMP was determined using the BIOTRAK™ cGMP enzyme immunoassay kit (GE Healthcare, United Kingdom). Ammonia was determined with the Ammonia Test Kit Ⅱ (Arkay, Inc., Kyoto, Japan). 3-nitrotyrosine in serum was determined by HPLC as in reference[15].

Blood flow determination by arterial spin labelling

Images were collected using a 3 Tesla MR scanner (Achieva 3T-TX, Philips, the Netherlands). Pulsed ASL was performed using EPISTAR strategy for tagging and control images with 150 mm thick slab of tagging pulse 30 mm far from the imaging region. The plane resolution was 3.5 mm × 3.5 mm. Multi-Slice (10 slices) multiphase (11 phases) acquisition was performed with a phase interval of 350 ms. No rest slabs or fat suppression pulses were applied during acquisition. Thirty repetitions were acquired in order to increase the signal to noise ratio. ASL data were processed using homemade analysis software written in Interactive Data Language 6.3 (Research System, Inc.). This analysis program fit the multi-phase data of every pixel to the Günther model[25] for a lock-looker ASL acquisition, and estimation of blood flow was performed with equation [A2] from reference[25]. The analysis program calculated automatically the M0 image from ASL data. After image analysis CBF maps are generated.

Statistical analysis

Values are given as mean ± SEM. Results were analysed by one-way ANOVA followed by post-hoc Bonferroni's multiple comparison test. Differences between groups were analyzed by Games-Howell test for multiple comparisons in the parameters with non-homogeneous variances. Adjusted *P* values are shown. Variables that were not previously age-adjusted (bimanual coordination and visuo-motor coordination) were analysed using univariate analysis of covariance (ANCOVA) with age included as covariate, followed by post-hoc Bonferroni. Bivariate correlations among variables were evaluated using the Pearson correlation test. The diagnostic performance for MHE was assessed using a logistic regression analysis, followed by receiver operating characteristic (ROC) curve to determine sensitivity and specificity and to identify the optimal threshold value. Analyses were performed using SPSS Version 19.0 (SPSS Inc, Chicago, United states) and two-sided *P* values < 0.05 were considered significant.

RESULTS

Sixteen (40%) of the 40 patients showed MHE according to PHES performance. The composition of groups, age, analytical data and aetiology of the disease are in Table 1.

Blood flow is increased in cerebellum and reduced in occipital lobe in MHE

BF was increased in cerebellar hemisphere and vermis in patients with MHE and reduced in occipital lobe (Figure 1). BF in cerebellar hemisphere was 22 ± 2 mL/min per 100 *g* in controls, 32 ± 3 mL/min per 100 *g* in patients without MHE ($P = 0.02$) and increased further in patients with MHE (36 ± 5 mL/min per 100 *g*) compared to control group ($P = 0.03$). BF in vermis were 38 ± 5 and 51 ± 5 mL/min per 100 *g* in controls and cirrhotics without MHE respectively, and increased in patients with MHE (75 ± 11 mL/min per 100 *g*) respect to control group ($P = 0.012$) and to patients without MHE ($P = 0.03$). In occipital lobe, BF was 16.6 ± 1.2 mL/min per 100 *g* in controls and 17.4 ± 1.7 mL/min per 100 *g* in cirrhotics without MHE, and decreased in patients with MHE (11.7 ± 1.1 mL/min per 100 *g*) compared to controls ($P = 0.017$) and to patients without MHE ($P = 0.02$).

MHE increases blood cGMP, 3-nitrotyrosine, IL-6 and IL-18

Cirrhotic patients with or without MHE show increased blood levels of cGMP, NO metabolites, 3-nitrotyrosine, IL-6, IL-18 and ammonia (Table 2). The increase in cGMP ($P < 0.05$), 3-nitrotyrosine ($P < 0.0001$), IL-6 ($P = 0.001$) and IL-18 ($P < 0.05$) was higher in patients with than without MHE (Table 2).

Performance in the psychometric tests

Controls completed the bimanual coordination test in 1.73 ± 0.05 min. Patients without MHE needed 2.16 ± 0.05 min ($P = 0.05$) and patients with MHE 2.8 ± 0.1 min, more than controls ($P < 0.0001$) or patients without MHE ($P < 0.001$) (Figure 2A, Table 3). In the visuo-motor coordination test, controls completed the task in 2.15 ± 0.08 min. The score was not affected in patients without MHE (2.61 ± 0.08 min). Patients with MHE needed more time (3.9 ± 0.3 min, $P < 0.0001$) (Figure 2B, Table 3). In the congruent task of the Stroop test of selective attention, controls read 102 ± 5 words. Patients without MHE read 90 ± 2 words ($P = 0.03$) and patients with MHE even less words (76 ± 4) which was lower than for controls ($P < 0.0001$) and patients without MHE ($P = 0.009$) (Figure 2C, Table 3). In the neutral task controls named 75 ± 3 colours. Patients without MHE named 67 ± 2 colours ($P = 0.07$) and patients with MHE 54 ± 3 co-

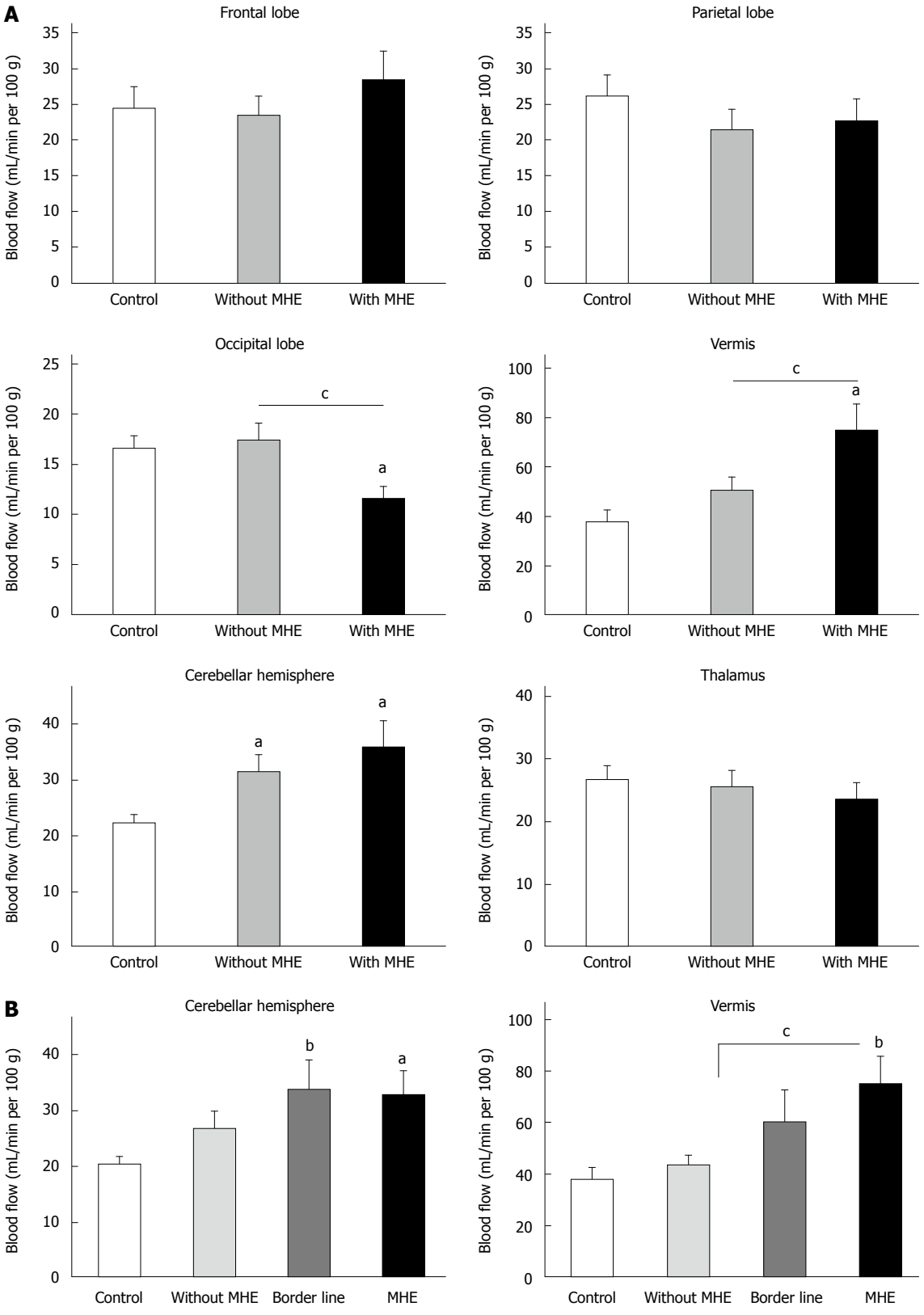

Figure 1 Blood flow in the different brain regions studied. A: Blood flow in frontal lobe, parietal lobe, occipital lobe, vermis, cerebellar hemisphere and thalamus in controls and cirrhotic patients without and with minimal hepatic encephalopathy (MHE); B: Blood flow in cerebellar hemisphere and vermis including the patients (n = 7) showing Psychometric Hepatic Encephalopathy Score (PHES) = -3, considered as borderline. Data are expressed as mL of blood per minute per 100 g of brain tissue (mean ± SEM) of 14 controls, 24 patients without and 16 with MHE. $^{a}P < 0.05$; $^{b}P < 0.01$ *vs* control, Values significantly different between patients with and without MHE are indicated by $^{c}P < 0.05$.

Table 2 Biochemical parameters in blood (mean ± SE)

Parameter	Controls (n = 14)	Patients without MHE (n = 24) P *vs* control	Patients with mHE (n = 16) P *vs* control	Patients with MHE P *vs* without MHE	P values
cGMP in plasma (pmol/mL)	0.64 ± 0.1	6.1 ± 0.6 P < 0.0001	8.7 ± 1.1 P < 0.0001	< 0.05	< 0.0001
Nitrates + Nitrites (μmol/L)	18 ± 1	38 ± 4 P = 0.004	44 ± 9 P = 0.004	NS	0.001
3-Nitro-tyrosine (nmol/L)	3.5 ± 1.2	10 ± 1	43 ± 6 P < 0.0001	< 0.0001	< 0.0001
IL-6 (pg/mL)	0.7 ± 0.2	2.3 ± 0.3 P < 0.05	4.4 ± 0.5 P < 0.0001	0.001	< 0.0001
IL-18 (pg/mL)	103 ± 13	268 ± 39 P = 0.02	404 ± 44 P < 0.0001	< 0.05	< 0.0001
Ammonia (μmol/L)	39 ± 4	84 ± 14 P = 0.04	103 ± 33 P = 0.006	NS	0.005

One-way analysis of variance (ANOVA) followed by post-hoc Bonferroni's multiple comparisons test were performed. Adjusted P values are shown. NS: Not significant.

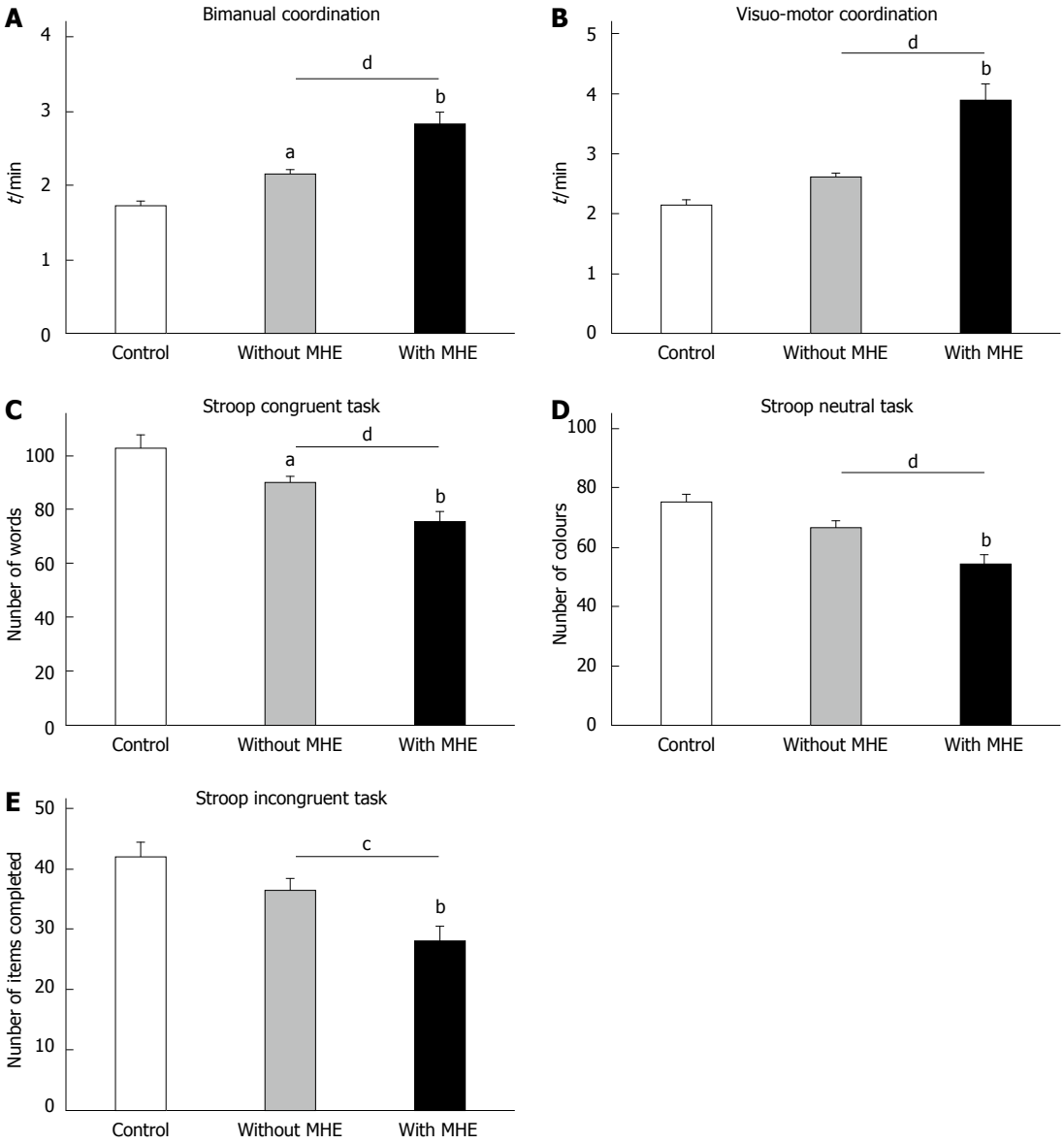

Figure 2 Performance in the Stroop, bimanual and visuo-motor coordination tests. A: Bimanual coordination test; B: Visuo-motor coordination test; C: Stroop congruent task; D: Stroop neutral task; E: Stroop incongruent task. Values are the mean ± SEM of 14 controls, 24 patients without and 16 with MHE. [a]P < 0.05; [b]P < 0.01 *vs* control, Values significantly different between patients with and without MHE are indicated by [c]P < 0.05; [d]P < 0.01.

Table 3 Psychometric tests and attention and coordination tasks in the different groups of cirrhotic patients and in controls (mean ± SD)

Test	Sub-test	Controls (n = 14)	Patients without MHE (n = 24)	Patients with MHE (n = 16)	Patients with MHE	P values
			P *vs* Control	P *vs* control	P *vs* without MHE	
CFF		43.2 ± 0.7	40.5 ± 0.7	36 ± 1	P = 0.001	< 0.001
				P < 0.001		
PHES Global score		-0.13 ± 0.25	-1.23 ± 0.30	-6.31 ± 0.72		
				P < 0.001	P < 0.001	< 0.001
	Symbol digit test	-0.07 ± 0.12	-0.11 ± 0.08	-0.87 ± 0.12		
				P < 0.001	P < 0.001	< 0.001
	NCT-A	-0.07 ± 0.15	-0.04 ± 0.07	-1.19 ± 0.28		
				P < 0.001	P < 0.001	< 0.001
	NCT-B	-0.07 ± 0.18	-0.27 ± 0.14	-2.07 ± 0.30		
				P < 0.001	P < 0.001	< 0.001
	Serial dotting test	0.00 ± 0.00	-0.57 ± 0.17	-1.19 ± 0.29	NS	
				P = 0.001		0.002
	Line tracing test	-0.07 ± 0.07	-0.15 ± 0.07	-1.25 ± 0.33	P < 0.001	
				P < 0.001		< 0.001
Stroop	Congruent task	102 ± 5	90 ± 2	76 ± 4		
			P = 0.03	P < 0.0001	P = 0.009	< 0.0001
	Neutral Task	75 ± 3	67 ± 2	54 ± 3		
				P < 0.0001	P = 0.003	< 0.0001
	Incongruent Task	42 ± 2	36 ± 2	28 ± 2	P = 0.030	0.001
				P = 0.001		
Bimanual coordination		1.73 ± 0.05	2.16 ± 0.05	2.83 ± 0.15		
			P = 0.05	P < 0.0001	P < 0.0001	< 0.0001
Visuo-motor coordination		2.15 ± 0.08	2.61 ± 0.08	3.89 ± 0.28		< 0.0001
				P < 0.0001	P < 0.0001	

Statistical analysis: One-way analysis of variance (ANOVA) followed by post-hoc Bonferroni's multiple comparisons test , except for Bimanual and Visuo-motor coordination tests, in which univariate analysis of covariance (ANCOVA) was performed, with age included as covariate, followed by post-hoc Bonferroni. Adjusted P values are shown. Stroop test: Congruent task: number of words read in 45 s; Neutral task: Number of colours read in 45 s; Incongruent task: Number of items completed in 45 s. Bimanual and Visuo-motor coordination tests: Score in minutes. CFF: Critical Flicker Frequency; PHES: Psychometric Hepatic Encephalopathy Score; NCT-A, NCT-B: Number Connection Test A and B: respectively; NS: Not significant.

lours, which was lower than for controls (P < 0.0001) and patients without MHE (P = 0.003) (Figure 2D, Table 3). In the incongruent task, controls named 42 ± 2 colours. Patients without MHE 36 ± 2 colours and patients with MHE 28 ± 2 colours, which was lower than for controls (P = 0.001) and patients without MHE (P = 0.03) (Figure 2E, Table 3). Performance in the 5 individual tests of the PHES battery is shown in Table 3.

Correlation analysis between performance in each psychometric test and BF in each brain area

BF in cerebellar hemisphere and vermis correlated with performance in most tests of PHES (NCT-A, NCT-B and line tracing test) and in the congruent task of Stroop test. BF in frontal lobe correlated with NCT-A (Table 4). Performance in bimanual and visuomotor coordination tests correlated only with BF in cerebellar hemisphere. BF in occipital lobe correlates with global performance in the PHES battery and with CFF but not with any individual psychometric tests (Table 4). Global performance in the PHES correlated with BF in frontal lobe (P = 0.007, r = -0.36), occipital lobe (P = 0.031, r = 0.309), cerebellar hemisphere (P = 0.01, r = -0.348), and especially in vermis (P < 0.001, r = -0.504).

Correlation analysis between BF in each area and biochemical parameters

BF in cerebellar hemisphere correlates with plasma cGMP and NO metabolites (Table 4). BF in vermis cerebellar also correlates with NO metabolites and with 3-nitrotyrosine. IL-18 in plasma correlates with BF in thalamus and occipital lobe (Table 4).

Blood flow in cerebellar vermis is predictive for MHE

To assess whether altered BF may be useful to predict MHE we performed logistic regression analyses. Univariate logistic regression using the presence of MHE as the dependent variable and blood flow in vermis as independent variable shows that BF significantly predicts MHE, with an OR of 1.042 (95%CI: 1.006-1.078, P = 0.021).

ROC curve analysis were performed to determine the cut-off, the area under the curve (AUC), and specificity and sensitivity for MHE detection. The ROC curve has an AUC of 0.714 (95%CI: 0.54-0.88, P = 0.027) for BF in vermis. At the cut-off of 46 mL/min per 100 g, the specificity was 57% and the sensitivity was 69%. Although these values are not excellent for diagnosis, the data show that BF in cerebellum is altered in patients with MHE and also in some patients without MHE who

Table 4 Correlations between tests and biochemical parameters and blood flow in the brain areas

Studied parameter/test	Sub-test	Frontal lobe	Parietal lobe	Occipital	Vermis	Cerebellar hemisphere	Thalamus
CFF		NS	NS	r = 0.353 P = 0.017	r = -0.326 P = 0.025	NS	NS
PHES Global score		r = -0.36 P = 0.007	NS	r = 0.309 P = 0.031	r = -0.504 P < 0.001	r = -0.348 P = 0.01	NS
	Symbol digit test	NS	NS	NS	NS	NS	NS
	NCT-A	r = -0.469 P < 0.001	NS	NS	r = -0.530 P < 0.001	r = -0.320 P = 0.018	NS
	NCT-B	NS	NS	NS	r = -0.531 P < 0.001	r = -0.307 P = 0.027	NS
	Serial dotting test	NS	NS	NS	NS	NS	NS
	Line tracing test	NS	NS	NS	r = -0.330 P = 0.018	r = -0.364 P = 0.007	NS
STROOP	Congruent task	NS	NS	NS	r = -0.320 P = 0.027	r = -0.354 P = 0.012	NS
	Neutral task	NS	NS	NS	NS	NS	NS
	Incongruent task	NS	NS	NS	NS	NS	NS
Bimanual coordination		NS	NS	NS	NS	r = 0.362 P = 0.011	NS
Visuo-motor coordination		NS	NS	NS	NS	r = 0.335 P = 0.017	NS
cGMP in plasma (pmoles/mL)		NS	NS	NS	NS	r = 0.301 P = 0.034	NS
Nitrates + Nitrites (mmol/L)		NS	NS	NS	r = 0.339 P = 0.043	r = 0.362 P = 0.023	NS
3-Nitro-tyrosine (nmol/L)		NS	NS	NS	r = 0.358 P = 0.011	NS	NS
IL-6 (pg/mL)		NS	NS	NS	NS	NS	NS
IL-18 (pg/mL)		NS	NS	r = -0.353 P = 0.041	NS	NS	r = -0.405 P = 0.032
Ammonia (mmol/L)		NS	NS	NS	NS	NS	NS

PHES: Psychometric Hepatic Encephalopathy Score; NCT-A, NCT-B: Number Connection Test A and B, respectively; CFF: Critical flicker frequency; NS: No significant correlation.

also show impaired bimanual coordination. This supports that altered BF in cerebellum detects some motor deficits earlier that the PHES battery. To get further insight on this matter, we sub-classified the patients showing a PHES of -3 (who are usually classified as without MHE) as "borderline" patients. There were 7 patients with PHES-3. BF in vermis was 60 ± 12 mL/min per 100 *g*, which was nearly significantly different (P = 0.055) from controls (Figure 1B). BF in cerebellar hemisphere of these "borderline patients" was 37 ± 6 mL/min per 100 *g* which was significantly different (P < 0.01) from controls and not different from patients with MHE (Figure 1B). This supports that altered BF in cerebellum detects some motor deficits earlier than the PHES battery in its present form.

DISCUSSION

The results reported show that CBF is increased selectively in the cerebellar vermis and reduced in the occipital lobe in patients with MHE compared with patients without MHE. CBF was increased in cerebellar hemispheres in cirrhotic patients irrespective of the presence of MHE as defined according to PHES. This suggests that (1) cerebellar hemisphere is the most sensitive region in cirrhotic patients; and (2) non invasive determination of BF in cerebellum using ASL would be useful for early detection of MHE even before it is detectable with PHES.

Treatment of HE is associated with lower hospitalization frequency and duration, better clinical status, and fewer adverse events[26]. We show that non invasive BF determination in cerebellum by ASL would allow early detection and treatment of MHE. This would reduce societal costs by reducing the number of motor vehicle accidents[27], improve quality of life and prevent progression to overt HE.

The data reported show that some motor alterations may appear before MHE is detectable using the PHES battery. This agrees with previous reports suggesting that impairment of some motor coordination functions are early markers for cerebral dysfunction in some patients with MHE even prior to neuropsychometric alterations becoming detectable[4]. These motor functions are mainly modulated in cerebellum, supporting that cerebellar alterations would contribute to these early alterations.

In addition to increased BF in cerebellar hemisphere, patients with MHE also show increased BF in cerebellar vermis and reduced BF in occipital lobe. Changes in BF in specific cortical areas are sensitive early markers of progression of mild cognitive impairment in Alzheimer's disease[28].

Changes in regional BF could also serve as markers of the presence and progression of MHE. Altered BF may contribute to neurological impairment and to impair performance in the PHES and in bimanual and visuo-motor coordination and/or in attention tests. BF in cerebellar hemisphere correlates with performance in most tests, suggesting that increased BF in cerebellar hemisphere may be an early relevant contributor to neurological impairment in MHE.

BF in cerebellar hemisphere is also increased in cirrhotic patients without MHE, who also show impaired bimanual coordination and performance in the congruent task in the Stroop test. This suggests that increased BF in cerebellar hemisphere is an early event which may contribute to neurological deterioration and reduced performance in bimanual coordination and the congruent Stroop task and predisposing to impair visuomotor coordination, executive functioning (NCT-B) and cognitive processing speed (NCT-A).

BF in cerebellar hemisphere and vermis correlate with performance in NCT-A and NCT-B (also known as trail making test). NCT-B examines executive functioning and NCT-A cognitive processing speed[29]. Cerebellar alterations result in impaired executive function and performance in NCT test in schizophrenia[30,31], Parkinsonism[32] or Cerebellar Cognitive Affective Syndrome[33]. Altered BF in cerebellum may contribute to impair executive functioning and performance in NCT tests in MHE.

Bimanual coordination correlates with BF in cerebellar hemisphere but not in other areas. This agrees with functional magnetic resonance imaging studies showing that cerebellum is a critical site for the control of bimanual coordination[34] and that intercerebellar coupling is key for execution of simultaneous bimanual movements[35]. Moreover, both bimanual coordination and BF in cerebellar hemisphere are impaired in patients without MHE according to PHES, supporting the idea that altered BF in cerebellar hemisphere would contribute to impair bimanual coordination.

The data reported show that patients which are classified as "without MHE" according to the PHES battery have significantly reduced bimanual coordination. We have previously shown that patients who do not show MHE as detected using the PHES already have some psychomotor slowing and impaired bimanual coordination, indicating that some mild neurological alterations are not detected with the PHES but can be detected by more sensitive procedures[5]. Butz *et al*[4] also showed that ataxia, tremor, and slowing of finger movements are early markers for cerebral dysfunction in at least a subgroup of cirrhotic patients even prior to alterations in performance in the PHES become detectable. These data suggest that, although the PHES battery is currently the gold standard for detection of the presence of MHE, and it has been very useful to unify the assessment of neurological alterations in cirrhotic patients, is not sensitive enough to detect some mild alterations in motor coordination and mental processing speed.

Alterations in both bimanual coordination and BF in cerebellum are early events, occurring before altered performance in the PHES, this suggests that bimanual coordination tests should be also performed for early MHE detection and that non invasive determination of BF in cerebellum using ASL would detect MHE earlier than the currently used PHES battery.

Visuo-motor coordination also correlates with BF in cerebellar hemisphere but not in other areas. This could be expected, as cerebellum is crucial in visuomotor coordination[36]. This further supports that many functions modulated by cerebellum are affected in patients with MHE and that cerebellum is more susceptible than other areas in cirrhotic patients.

Magnetic resonance studies show that cerebellum is involved in the Stroop task[37]. Nabeyama *et al*[37] showed that in patients with obsessive-compulsive disorder reduced performance in the Stroop test is associated with reduced cerebellum activation. Moreover, patients with Cerebellar Cognitive Affective Syndrome show reduced performance in the Stroop test[33]. This suggests that altered BF in cerebellum could also contribute to impair performance in the Stroop test in patients with MHE.

To shed some initial insight on possible mechanisms involved in altered CBF in each area in MHE we also assessed the correlations between CBF and some biochemical parameters in blood. Ammonia or IL-6 levels do not correlate with CBF in any area, suggesting that hyperammonemia and IL-6 related inflammation are not main direct contributors to alterations in CBF. Serum IL-18 correlates with CBF in occipital lobe and thalamus, but not in cerebellum. This suggests that some inflammatory factors would contribute to alter CBF in these areas but not in cerebellum.

CBF in cerebellar hemisphere correlates with NO metabolites and cGMP, suggesting an association between alterations in the NO-cGMP system and in BF in cerebellum but not in thalamus or cortex. This agrees with the role of NO in CBF modulation. Neuronal NO plays an essential role in coupling neuronal activity with CBF in cerebellum but not in cortex[38,39]. NO modulates BF in cerebellum via stimulation of soluble guanylyl cyclase and cGMP formation[38-40]. This suggests that altered NO-cGMP pathway could contribute to increase BF in cerebellum in patients with MHE and even in cirrhotic patients without MHE.

The NO-cGMP pathway is strongly altered in blood[25,41-43] and cerebellum[44] of cirrhotic patients with MHE or died in HE. Moreover, altered activation of guanylate cyclase by NO in lymphocytes correlates with the MHE grade in cirrhotic patients. It has been suggested that altered NO-cGMP pathway contributes to cognitive impairment in MHE[42]. The contribution of altered NO-cGMP pathway in cerebellum to cognitive impairment has been clearly established in animal models of MHE. Rats with MHE have reduced ability to learn a Y maze task, which is a consequence of impaired function of the glutamate-NO-cGMP pathway in cerebellum. Learning

ability is restored by treatments that restore the pathway and cGMP levels[12-14,45].

These data suggest the idea that, in patients with MHE, altered NO-cGMP pathway is associated with altered CBF in cerebellum which, in turn, may contribute to induce MHE, impairing bimanual and visuo-motor coordination, executive functioning, cognitive processing speed and performance in the PHES. If this were the case, treatments normalizing the NO-cGMP pathway could improve CBF in cerebellum and cognitive function in cirrhotic patients with MHE.

In summary, the data support that: (1) CBF is more sensitive in cerebellum than in other areas of cirrhotic patients; (2) altered CBF in cerebellum is an early event contributing to initiate neurological deterioration, impairing bimanual coordination and the congruent Stroop task and predisposing to impair visuomotor coordination, executive functioning and cognitive processing speed; (3) altered NO-cGMP pathway would contribute to alter CBF in cerebellum; and (4) non invasive determination of CBF in cerebellum using ASL may detect MHE earlier than the currently used PHES battery.

COMMENTS

Background

Patients with minimal hepatic encephalopathy (MHE) show neurological impairment in specific tasks to which selective regional alterations in blood flow (BF) could contribute. Invasive positron emission tomography and single photon emission computed tomography techniques have shown altered cerebral blood flow patterns in cirrhotic patients, but there is a large variability between the results published. The possible contribution of alterations in BF in specific brain areas to neurological alterations in patients with MHE remains unclear. Previous reports suggest that impairment of some motor coordination functions are early markers for cerebral dysfunction in some patients with MHE even prior to neuropsychometric alterations becoming detectable. These motor functions are mainly modulated in cerebellum, supporting that cerebellar alterations would contribute to these early alterations.

Research frontiers

Arterial spin labeling (ASL) is a non-invasive magnetic resonance technique that measures quantitatively cerebral perfusion by magnetically labeling protons in arterial blood water. This study assess whether non invasive BF measurement by ASL in several brain regions detects MHE and/or specific neurological alterations.

Innovations and breakthroughs

The authors analyzed BF by ASL in different brain areas of controls and cirrhotic patients without and with MHE. The authors found that BF is more affected in cerebellum than in other areas of cirrhotic patients and that BF determination in cerebellum using ASL may detect MHE earlier than the Psychometric Hepatic Encephalopathy Score (PHES) battery. Altered NO-cGMP pathway seems to be associated to altered BF in cerebellum.

Applications

ASL technique would be useful to detect MHE and/or specific neurological alterations. This would allow early treatment and follow-up of the eficiency of therapeutic treatments.

Terminology

ASL is a non-invasive magnetic resonance technique that measures quantitatively cerebral perfusion by magnetically labeling protons in arterial blood water. PHES is a battery of five psychometric tests that has been recommended as the "gold standard" in the diagnosis of MHE. NO-cGMP pathway is an intracellular signaling pathway involved in important brain functions.

Peer review

In this cross-sectional study, the authors report a moderate correlation between non-invasive cerebral blood flow, in the cerebellum and minimal hepatic encephalopathy as well as other psychometric tests in patients with cirrhosis. Overall, this is a rigorous and well-conducted study.

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RETROSPECTIVE STUDY

Surgical failure after colonic stenting as a bridge to surgery

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Abstract

AIM: To identify risk factors for surgical failure after colonic stenting as a bridge to surgery in left-sided malignant colonic obstruction.

METHODS: The medical records of patients who underwent stent insertion for malignant colonic obstruction between February 2004 and August 2012 were retrospectively reviewed. Patients with malignant colonic obstruction had overt clinical symptoms and signs of obstruction. Malignant colonic obstruction was diagnosed by computed tomography and colonoscopy. A total of 181 patients underwent stent insertion during the study period; of these, 68 consecutive patients were included in our study when they had undergone stent placement as a bridge to surgery in acute left-sided malignant colonic obstruction due to primary colon cancer.

RESULTS: Out of 68 patients, forty-eight (70.6%) were male, and the mean age was 64.9 (range, 38-89) years. The technical and clinical success rates were 97.1% (66/68) and 88.2% (60/68), respectively. Overall, 85.3% (58/68) of patients underwent primary tumor resection and primary anastomosis. Surgically successful preoperative colonic stenting was achieved in 77.9% (53/68). The mean duration, defined as the time between the SEMS attempt and surgery, was 11.3 d (range, 0-26 d). The mean hospital stay after surgery was 12.5 d (range, 6-55 d). On multivariate analysis, the use of multiple self-expanding metal stents (OR = 28.872; 95%CI: 1.939-429.956, P = 0.015) was a significant independent risk factor for surgical failure of preoperative stenting as a bridge to surgery. Morbidity and mortality rates in surgery after stent insertion were 4.4% (3/68) and 1.5% (1/68), respectively.

CONCLUSION: The use of multiple self-expanding metal stents appears to be a risk factor for surgical failure.

Key words: Colorectal neoplasms; Endoscopy; Intestinal obstruction; Risk factors; Stents

Core tip: When self-expanding metal stents (SEMS) is used as a bridge to surgery, the goal is a successful surgical outcome. When surgical results are not good after colonic stenting in patients with malignant colonic obstruction (MCO), many physicians have wondered about the risk factors of surgical failure and wanted to improve their results. Our results show that the use of multiple SEMS was an independent risk factor for surgical failure on multivariate analysis. The identification of this risk factor might help physicians make decisions

regarding an appropriate modality for patients with acute left-sided MCO and should provide a foundation for establishing a consensus on treatment strategies in these patients.

Kim JH, Kwon KA, Lee JJ, Lee WS, Baek JH, Kim YJ, Chung JW, Kim KO, Park DK, Kim JH. Surgical failure after colonic stenting as a bridge to surgery. *World J Gastroenterol* 2014; 20(33): 11826-11834 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i33/11826.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i33.11826

INTRODUCTION

Approximately 8%-29% of colon cancer patients present with obstructive symptoms on diagnosis[1]. Malignant colonic obstruction (MCO) requires urgent decompression because it may result in colonic necrosis and perforation due to colonic mucosal friability, which is the result of massive colon distension[2].

Conventionally, MCO has been managed by emergency surgical procedures, including loop colostomy followed by colostomy reversal. The formation of a stoma is known to have a negative impact on aspects of health-related quality of life in patients, and the stoma is eventually left unclosed in many patients[1,3,4]. Furthermore, emergency colonic surgery in this setting often leads to higher mortality (8%-24%), morbidity (46%-62%), and incidence of stoma retention than elective surgery due to the poor general condition and lack of bowel preparation of the patient[2,3,5,6].

It is necessary, therefore, to identify other alternatives for MCO decompression in order to avoid emergency surgery. One effective method is the use of self-expanding metal stents (SEMS) in patients with MCO. Previous reports have offered strong evidence of their advantages, such as high effectiveness and fewer complications[7-10]. Recently, however, increased emphasis has been placed on using colonic stents as a bridge to surgery (BTS). Additionally, there is an increasing controversy regarding the efficacy and long-term outcomes of stenting as a BTS compared to emergency surgery for MCO[3,11].

When a SEMS is used as a BTS, the goal is the achievement of a successful surgical outcome. However, there has been no report on the predictors of poor surgical outcomes after stenting as a BTS. The aim of this study was to identify the risk factors for surgical failure after colonic stenting as a BTS in patients with acute left-sided MCO.

MATERIALS AND METHODS

Patients

The medical records of patients who underwent SEMS placement for MCO at Gachon University Gil Medical Center (Incheon, Korea) from February 2004 to August 2012 were retrospectively reviewed.

SEMS as a BTS was attempted in eligible patients with acute left-sided MCO. Patients with MCO had overt clinical symptoms and signs of obstruction. MCO was diagnosed by computed tomography (CT) and colonoscopy, whereas CT confirmed the presence of obstructive lesions by identifying upstream colonic dilatation, with colonoscopic confirmation if an inability to pass the endoscope proximally was noted.

A total of 181 patients underwent SEMS insertion under endoscopic and fluoroscopic guidance during the study period. Patients were excluded if they fell within any of the following categories: stenting for palliation (n = 55) or for obstruction caused by metastatic colon cancer (n = 20) or a benign disease (n = 1); anastomosis site stenosis (n = 7); refusal of further treatment and evaluation or loss during follow-up (n = 12); right colonic obstruction (n = 15); distal tumor margin of less than 10 cm from the anal verge (n = 2); and partial obstruction allowing endoscopic passage (n = 1). A total of 68 consecutive patients were included in our study after having undergone SEMS placement as a BTS in acute left-sided MCO due to primary colon cancer (Figure 1). The study was approved by the Institutional Review Board of Gachon University Gil Medical Center (IRB No. GDIRB2013-09).

Procedure details

Informed consent was obtained from patients after an adequate explanation of the procedure. Enema was performed in all patients several hours before the procedure. A two-channel therapeutic endoscope (GIF-2T240; Olympus Optical Corp., Tokyo, Japan) or colonoscope (CF-Q240L; Olympus) was used. For stenting, ComVi Enteral Colonic Stents (Taewoong Medical Co., Seoul, South Korea) and Niti-S Enteral Colonic Stents (Taewoong Medical Co.) were used.

As previously reported[7,10,12], SEMS were inserted by the following process. The colonoscope was first introduced to the obstruction site, followed by the passage of a guidewire through the stricture under endoscopic and fluoroscopic guidance. After measuring the length of the stricture with CT and/or barium enema and/or fluoroscopy using water soluble contrast, a stent at least 3 cm longer than the stricture was chosen to adequately bridge the stricture. Covered or uncovered stents were selected depending on the endoscopists' preference. To confirm the appropriate stent positioning and expansion, repeated simple radiograms were made during hospitalization. Balloon dilatation of the stricture was not performed.

After SEMS insertion as a BTS, a pre-operative evaluation was performed, and the time of elective surgery was determined by the attending surgeon following an assessment of the patient's bowel function and clinical condition. Two surgeons specializing in coloproctology performed the operations, and the type of procedure was determined by the surgeon depending on the location of the primary disease and the intraoperative con-

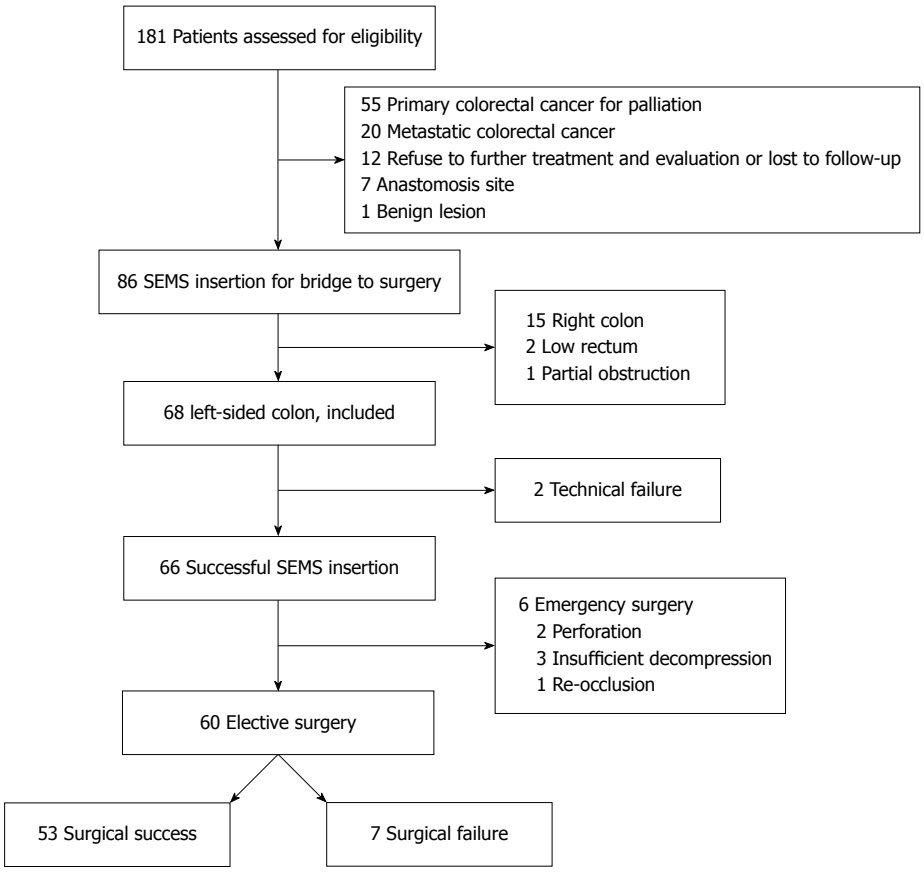

Figure 1 Flowchart showing patient selection in this study. SEMS: Self-expanding metal stents.

ditions of the patient. Patients with successful SEMS placement were deemed eligible for elective surgery and received a bowel preparation using 2-4 liters of polyethylene glycol in the evening before surgery and a single dose of first-generation cephalosporin for antibiotic prophylaxis 30 min before anesthetic induction. At the surgeons' discretion, continuous antibiotic therapy was employed as deemed necessary. Primary tumor resection and primary anastomosis (PTRPA) were performed when possible.

Definition

The technical success rate was defined as the ratio of patients with correctly placed SEMS upon stent deployment across the entire stricture length to the total number of patients. The clinical success rate was defined as the ratio of patients with technical success and successful maintenance of stent function before elective surgery, regardless of the number of SEMS deployed to the total number of patients.

The surgical success rate of SEMS as a BTS was defined as the ratio of patients with successful surgical outcomes, such as successful PTRPA, to the total number of patients. Unsuccessful surgical outcomes were defined as the failure of PTRPA or subtotal/total colectomy due to insufficient colonic decompression. Surgical failure was thus inclusive of technical failure, unplanned surgery, and total/subtotal colectomy due to insufficient decompression.

PTRPA was defined as the surgical procedure used to reconnect two sections of the colon following primary resection of the tumor. Multiple SEMS was defined as having at least two stents deployed at the first session or undergoing more than two stenting sessions.

Statistical analysis

Statistical analysis was carried out using SPSS 12.0 (IBM SPSS Statistics, IBM Corporation, Armonk, NY) for MS Windows®. Continuous data are presented as means (range) and categorical data as absolute numbers and percentages. For univariate analysis, continuous data were analyzed using the independent *t*-test, and other categorical data were analyzed using the χ^2 or Fisher's exact test. Multivariate analysis by logistic regression was performed using the statistically significant variables found in the univariate analysis. Two-tailed *P*-values of 0.05 or less were considered to indicate statistical significance.

RESULTS

Baseline characteristics

The baseline data for the patients are summarized in Table 1. Out of 68 patients, forty-eight (70.6%) were male, and the mean age was 64.9 years (range, 38-89 years). None had received prior chemotherapy, and each

Table 1 Baseline demographic and clinical characteristics of patients *n* (%)

	Total (*n* = 68)
Age (yr)	64.9 (38-89)
Male/female	48 (70.6)/20 (29.4)
BMI (kg/m^2)	22.9 (17.7-31.9)
Previous IA surgery	19 (27.9)
Laboratory findings	
Hemoglobin (g/dL)	12.4 (7.2-17.3)
White blood cells (/mm^3)	8471.7 (3050-19850)
Albumin (g/dL)	3.8 (2.5-5.2)
ASA classification	
1	11 (16.2)
2	50 (73.5)
3	7 (10.3)
TNM Stage of tumor	
Ⅱ	22 (32.4)
Ⅲ	26 (38.2)
IVA	20 (29.4)
Histology	
WD	8 (11.8)
MD	57 (83.8)
PD	3 (5.6)
Location of obstruction	
Sigmoid flexure	5 (7.4)
Descending colon	7 (10.3)
Sigmoid-descending area	9 (13.2)
Sigmoid colon	22 (32.4)
Recto-sigmoid area	25 (36.8)

BMI: Body mass index; IA: Intra-abdominal; ASA: American society of anesthesiologists; WD: Well-differentiated cancer; MD: Moderate-differentiated cancer; PD: Poorly-differentiated cancer.

first presented as acute obstruction due to primary colon cancer. The most commonly obstructed site was the rectosigmoid junction (25/68, 36.8%), and the second most common site was the sigmoid colon (22/68, 32.4%). Covered SEMS were used in 50.0% (33/66) of patients with technical success. The mean duration, defined as the time between SEMS attempts and surgery, was 11.3 d (range, 0-26 d). The mean hospital stay after surgery was 12.5 d (range, 6-55 d).

Success rate

In the first attempt, 66 of 68 patients (97.1%) achieved a technically successful placement of SEMS. SEMS insertion was not successful in two patients, in one due to failure of guide wire passage through the completely obstructed stricture (descending colon), and in the other, due to acute angulation of the colonic flexure, which caused difficulty in guide wire passage (rectosigmoid junction) (Table 2). They eventually underwent emergency surgery.

Clinical success was achieved in 88.2% (60/68) of patients. Clinical failure occurred in 8, who then required emergency surgery (Figure 1 and Table 2). Six patients, not counting the two who experienced technical failures, encountered clinical failure due to complications: two with perforation, one with re-obstruction, and three with insufficient decompression. The mean time from stent placement to surgery was 2.1 d (range, 0-8 d) in patients with clinical failure and 12.6 d (range, 4-26 d) in those with clinical success.

Successful PTRPA was achieved in 85.3% (58/68) of patients, including one following technical failure and 57 following technical success. In detail, failure in PTRPA occurred in five patients (5/8, 62.5%) following clinical failure and five (5/60, 8.3%) following clinical success.

In patients with clinical success, 7 patients did not achieve surgical success. In three, stent-related complications were the cause of surgical failure. The remaining four failed surgically due to unsuccessful surgical outcomes. The surgical success rate of SEMS as a BTS was 77.9% (53/68).

Complication and surgical outcomes

Complications were observed during the first 60 d after SEMS placement, including both stent-related and surgery-related complications (Tables 2 and 3).

Procedure-related complications were observed in 12 cases. Of these, two patients experienced perforation and underwent emergency surgery. The stent was observed to be *in situ* during elective surgery, except in two patients in whom clinically silent migration had occurred; they both underwent successful PTRPA. Another two patients with re-obstruction due to stool impaction underwent a second stent placement 1 and 3 d after the first stent insertion, respectively. One of the patients with re-obstruction was successfully re-stented 1 d after the first stent insertion; an emergency Hartmann's operation was subsequently required due to insufficient decompression. The other patient, who underwent stent reinsertion 3 d after the first stent insertion, received an elective operation; however, subtotal colectomy was required due to inadequate colonic decompression 13 d after the first stent placement. The remaining six patients with insufficient decompression included three who underwent emergency operations and three who underwent elective surgery.

Postsurgical complications occurred in 3/68 of patients; these patients underwent elective surgery (Table 3). These three patients experienced anastomosis leakage, wound infection, or pneumonia. The patient who underwent a segmental resection en bloc after successful stenting developed anastomotic leakage three days after surgery and had to undergo a re-operation for colostomy of the transverse colon. The patient with wound infection recovered after antibiotic treatment and was discharged 10 d after surgery. The patient who had pneumonia eventually passed away 6 d after elective surgery (left hemicolectomy); he had underlying medical conditions including hypertension and previous subarachnoid hemorrhage.

Univariate and multivariate analysis of risk factors for overall stent failure

Comparisons between patients with surgical failures and successes following technical success are summarized in Table 4. There were no differences between the two

Table 2 Etiology of surgical failure in patients undergoing stenting as a bridge to surgery *n* (%)

	Emergency surgery (*n* = 8)		Elective surgery (*n* = 60)		Total (*n* = 68)
	PTRPA(-) (*n* = 5)	PTRPA(+) (*n* = 3)	PTRPA(-) (*n* = 5)	PTRPA(+) (*n* = 55)	
Surgical failure					
Technical failure	1	1	0	0	2 (2.9)
Stent-related complications					
Perforation	1	1	0	0	2 (2.9)
Re-obstruction	1[1]	0	0	1[1,2]	2 (2.9)
Insufficient decompression	2	1	2[1]	0	5 (7.4)
Unsatisfactory surgical results					
PTRPA not feasible	0	0	3	0	3 (4.4)
Subtotal colectomy	0	0	0	1	1 (1.5)
Surgical success					
Stent-related complications					
Insufficient decompression	0	0	0	1[1]	1 (1.5)
Migration	0	0	0	2	2 (2.9)
Satisfactory surgical results				50	50 (73.5)

[1]Multiple SEMS was defined as a number of stent ≥ 2 at the first session or a session number ≥ 2; [2]Patients undergoing subtotal colectomy. PTRPA: Primary tumor resection and primary anastomosis.

Table 3 Types of and complications due to operations after colonic stenting as a bridge to surgery *n* (%)

Complications	Value
Type of operation (*n* = 68)	
Anterior resection	27 (39.7)
Left hemicolectomy	13 (19.1)
Low anterior resection	12 (17.6)
Hartmann's operation	7 (10.3)
Subtotal colectomy	3 (4.4)
Stoma creation	1 (1.5)
Quadrantectomy	1 (1.5)
Anterior resection with ileostomy	1 (1.5)
Total colectomy	1 (1.5)
Low anterior resection with ileostomy	1 (1.5)
Segmental resection en bloc	1 (1.5)
Surgery-related complications (*n* = 68)	
Wound infection	1 (1.5)
Pneumonia	1 (1.5)
Anastomosis leakage	1 (1.5)

Table 4 Comparison of factors related to surgical failure and success in patients achieving technical success *n* (%)

	Surgical failure (*n* = 13)	Surgical success (*n* = 53)	*P* value
Patient-related factor			
Age > 70 yr	6 (46.2)	20 (37.7)	0.578
Male	10 (76.9)	36 (67.9)	0.727
BMI < 18.5 kg/m^2	3 (23.1)	1 (1.9)	0.004
Previous IA surgery	3 (23.1)	16 (30.2)	0.612
Anemia	9 (69.2)	28 (52.8)	0.286
Leukocytosis	2 (15.4)	14 (26.4)	0.406
Hypoalbuminemia	6 (46.2)	11 (20.8)	0.061
ASA 1-2	12 (92.3)	47 (88.7)	0.703
Tumor-related factor			
WD and MD cancer	13 (100)	50 (94.3)	0.380
TNM stage Ⅲ-ⅣA	12 (92.3)	32 (60.4)	0.029
Obstructive site			0.511
Sigmoid flexure	0 (0)	5 (9.4)	
Descending colon	0 (0)	6 (11.3)	
Sigmoid-descending area	2 (15.4)	7 (13.2)	
Sigmoid colon	5 (38.5)	17 (32.1)	
Recto-sigmoid colon	6 (46.2)	18 (34.0)	
Flexure area	8 (61.5)	30 (56.6)	0.747
Stent-related factor			
Uncovered	6 (46.2)	27 (50.9)	0.757
Diameter ≤ 22 (mm)	9 (62.5)	34 (64.2)	0.731
Length ≥ 10 (cm)	8 (61.5)	27 (50.9)	0.493
Multiple SEMS	4 (30.8)	1 (1.9)	< 0.001
Interval from SEMS to surgery (d)	9.8 (0-26)	12.1 (4-26)	0.354
Hospital stay after surgery (d)	15.4 (7-44)	11.7 (6-55)	0.151

BMI: Body mass index; IA: Intra-abdominal; ASA: American society of anesthesiologists; WD: Well-differentiated cancer; MD: Moderate-differentiated cancer; SEMS: Self-expanding metal stents.

groups in terms of age, gender, or history of previous intra-abdominal surgery. Anemia (male < 13 g/dL, female < 12 g/dL) and hypoalbuminemia (albumin < 3.5 g/dL) were more common in the surgical failure group, and leukocytosis (> 10000/mm^3) was more common in the surgical success group, although there were no significant differences. Body mass index (BMI) < 18.5 kg/m^2 was significantly more common in the failure group (23.1% *vs* 1.9%, $P = 0.004$).

Advanced stages, defined as stages Ⅲ and ⅣA, were significantly more common in the surgical failure group than in the surgical success group (92.3% *vs* 60.4%, $P = 0.029$). The flexure area was more frequently obstructed in the surgical failure group than in the success group, but there was no significant difference.

Variables related to stent characteristics, including length or diameter of stent and stent type, were not identified as risk factors for surgical failure. However, the use of multiple SEMS was significantly more common in the surgical failure group (30.8% *vs* 1.9%, $P < 0.001$).

On multivariate analysis, only multiple SEMS was a significant risk factor correlating to surgical failure (OR = 28.872; 95%CI: 1.939-429.956, $P = 0.015$) (Table 5). In five patients who underwent multiple SEMS, only one patient (1/5, 20%) who received two SEMS in the

Table 5 Multivariate analysis of risk factors for surgical failure in patients achieving technical success

	OR	95%CI	*P* value
BMI			
⩾ 18.5	1 (reference)		
< 18.5	9.759	0.784-121.527	0.077
TNM stage			
Ⅱ	1 (reference)		
Ⅲ and IVA	7.685	0.666-88.722	0.102
Multiple SEMS			
No	1 (reference)		
Yes	28.872	1.939-429.956	0.015

BMI: Body mass index; SEMS: Self-expanding metal stents.

first session due to insufficient decompression met with surgical success (Table 2). Two of the remaining patients who underwent elective surgery received two SEMS in the first session due to insufficient decompression and eventually underwent Hartmann's operation. The remaining two patients underwent a second stenting session due to re-obstruction; one underwent emergency surgery, and the other, a subtotal colectomy.

DISCUSSION

In this study, the technical success rate was 97.1%, which is comparable to the rates of previous studies, which range from 70.7% to 96.2%, according to systematic reviews and pooled/meta-analyses[2,9,13]. The definition of a clinically successful stent deployment differed slightly among these studies. Several of the previous studies defined clinical success based on intestinal transit or flatus/stool passage within 1-3 d after the procedure[13-15]. In this study, clinical success was defined as the achievement of colonic decompression with stool passage between stent placement and elective surgery, which conforms to the intention of deploying colonic stents as a BTS. Based on this definition, the clinical success rate was 88.2% in this study, which is consistent with the reported rates of 69.0%-92.0%[2,16].

When a SEMS is used as a BTS, the purpose is to obtain a successful surgery that facilitates optimal outcomes and permits PTRPA. Therefore, unlike in other studies, surgical success, defined as the feasibility of PTRPA as elective surgery except for subtotal/total colectomy caused by poor bowel decompression, was also assessed. BTS stenting allowed for surgical success in 77.9% of all patients; this result is relatively high compared to the rates in the range of 55.3%-64.9% that have been reported in previous studies[17,18]. The reason for this may be that the denominator in the success rate of the SEMS group (defined as patients with SEMS insertion as a BTS) included patients with technical failure (who actually underwent emergency surgery) for analysis on an intention-to-treat basis, and the technical failure rates in other prospective studies were higher than in this study.

SEMS as a BTS showed conflicting outcomes in a recent meta-analysis and in randomized trials[2,14,18-20]. The endoscopists who participated in these trials had some differences in their levels of experience and skill[21,22]. Low technical success in SEMS placement might have been the main cause of their negative results for the deployment of SEMS. It would not be reasonable, therefore, to generalize the conclusions of some of the reports that SEMS is not advantageous as a BTS because an endoscopist's experience and skill are important factors in colonic stenting. For patients in whom SEMS placement was attempted as a BTS in MCO, previous studies have reported that the factors associated with technical failure included the severity of obstruction, the extra-colonic origin of tumor, the proximal colonic obstruction, and the presence of carcinomatosis[14,23]. In this study, the factors associated with technical failure could not be analyzed because the incidence of technical failure (2 cases) was too low.

In patients undergoing emergency surgery without SEMS insertion before surgery (the surgery group), the previously published stoma formation rates are 32%-57%[4,15,24]. When the SEMS group included patients with technical failure, the initial stoma formation rates for the SEMS group were 40%-53%[4,15]. No difference is apparent between the two groups. However, the primary stoma formation rate in the present study was 14.7% (10/68), with only 5 patients (5/60, 8.3%) requiring a diverting stoma in patients with clinical success. The remaining 5 patients (5/8, 62.5%) with clinical failure underwent stoma creation due to stent-related complications (n = 4) and technical failure (n = 1). This result suggests that BTS stenting is advantageous if SEMS insertion is technically successful and if function is well-maintained up to surgery[5,15].

The focus of this study was to identify risk factors for surgical failure in patients with technical success. The use of multiple SEMS was an independent risk factor for surgical failure on multivariate analysis. Currently, when strictures are not adequately covered or decompressed by a single SEMS or in a single session, the option is to immediately place a second stent or to undergo another session, as needed[25]. In the present study, out of the five patients who had undergone multiple SEMS placement, clinical success was achieved in four, allowing for elective surgery. However, only one patient achieved surgical success. Repeated attempts at colonic decompression using SEMS increased the clinical success rate, but the surgical success, the essential purpose of BTS stenting, was unachieved. Although the present study has found that the use of multiple SEMS is an independent risk factor for surgical failure, this may not necessarily mean that, upon the clinical failure of a single SEMS, surgery should be immediately considered as the next course of action. There is still no consensus in the literature regarding this relationship and further data is required to establish it.

When using stents for palliation of MCO, the stent length (⩾ 10 cm) and diameter (⩽ 22 mm) are reported to be risk factors for poor long-term outcomes

of SEMS[26,27]. Patient- and tumor-related factors and characteristics of SEMS were not identified as risk factors for surgical failure in the present study. BTS stenting differs from palliative stenting in that, for the former, the functionality of the SEMS must be maintained until elective surgery.

In this study, one case (1/68, 1.5%) of intra-procedural complications and eleven cases (11/68, 16.2%) of post-procedural complications were observed, which is comparable to the results of other studies on colonic stenting as a BTS with rates of 7%-24%[14,24,27]. Two patients in whom covered stents were inserted experienced migration. This may have been due to the smooth surface of the stent and their less severe colonic obstruction. Although the potential for tumor cell dissemination due to perforation is unclear, perforation is the most significant complication of SEMS[15] .Balloon dilation, specifically designed stents, and chemotherapy are considered contributing factors associated with stent-related perforation[8,28]. Two cases of perforation occurred in 68 patients in the present study. This result is lower than the 3%-9% reported in previously published studies[16,24,27,29], most likely because none of the patients in this study were associated with these factors.

Morbidity and mortality rates in surgery after SEMS insertion were 3/68 (4.4%) and 1/68 (1.5%), respectively, and are comparable to the respective reported rates of 0%-36% and 0%-9% from other studies on colonic stenting as a BTS[5,6,17,24]. All of these data represent patients who underwent elective surgery. However, it is difficult to analyze the contributing factors because of the small sample size and rarity of such events in this study.

The mean duration between successful stenting and elective surgery [12.1 d (range, 4-26 d)] was relatively longer than that reported in other studies[14,24], and the increase in the complication rates may have been due to this short interval (5-16 d). A sufficient interval between SEMS placement and surgery is required to allow for optimal decompression and improvement of the patient' s clinical condition[14,24]. However, there is no consensus on the optimal stent-to-surgery duration for bowel decompression and improvement under clinical conditions.

The beneficial efficacy of the SEMS as a BTS on right-sided colon cancer is obviously controversial. Obstructive right sided colon cancers can be treated by PTRPA, even in emergency settings, which is why we did not include right-sided obstructions in our study[30].

This is a retrospective study at a single tertiary center, and thus the possibility of selection bias cannot be ruled out. In addition, patients with severe obstruction would have been more likely to receive emergency surgery than stent insertion as a BTS. The confidence interval was so wide that the statistical power was not high enough to interpret and generalize the study results. Additionally, the use of multiple SEMS most likely suggests a difficult, severe stricture. This may be explained by the fact that the number of patients included in this study was relatively small. Despite these limitations, this study suggests that the use of SEMS is an effective BTS with an acceptable complication rate in most patients.

In conclusion, the finding that the use of multiple SEMS is an independent risk factor for surgical failure may aid in therapeutic decision-making between surgery and endoscopic stenting in patients with acute left-sided MCO. Larger, prospective studies on the clinical impact of multiple SEMS as a BTS appear to be necessary to confirm the findings presented in this report.

COMMENTS

Background

It is necessary to identify other alternatives for the decompression of malignant colonic obstruction (MCO) in order to avoid emergency surgery. One effective method is using self-expanding metal stents (SEMS) in patients with MCO. Previous reports have offered strong evidence of their advantages, such as high effectiveness and fewer complications. However, there has been no report on the predictors of poor surgical outcomes after stenting as a bridge to surgery (BTS).

Research frontiers

Most of the previous studies have been focused on the technical and clinical success of SEMS. However, when SEMS are used as a BTS, the goal is a successful surgery that enables the optimal outcome to be achieved and permits primary tumor resection and primary anastomosis (PTRPA). Therefore, unlike other studies, the aim of this study was to identify risk factors for surgical failure after colonic stenting as a BTS in patients with MCO.

Innovations and breakthroughs

The focus of this study was to identify the risk factors for surgical failure in patients with technical success. In this study, repeated attempts at colonic decompression using SEMS increased the clinical success rate, but surgical success, the essential purpose of BTS stenting, remained unachieved. These results constitute very important and interesting findings. Still, although the present study has found the use of multiple SEMS to be an independent risk factor for surgical failure, this may not necessarily mean that, upon the clinical failure of single SEMS, surgery should immediately be entertained as the next course of action.

Applications

The use of multiple SEMS was an independent risk factor for surgical failure, upon multivariate analysis. Currently, when strictures are not adequately covered or decompressed by a single stent or in a single session, the option is to immediately place a second stent or to undergo another session, as needed. These findings may aid in the therapeutic decision-making between surgery and endoscopic stenting in patients with acute left-sided MCO.

Terminology

The surgical success rate of SEMS as a BTS was defined as the ratio of patients with successful surgical outcomes, such as successful PTRPA, to the total number of patients. Unsuccessful surgical outcomes were defined as the failure of PTRPA or subtotal/total colectomy due to insufficient colonic decompression. Surgical failure was thus inclusive of technical failure, unplanned surgery, and total/subtotal colectomy due to insufficient decompression. Multiple SEMS was defined as having at least two stents deployed at the first session or undergoing more than two stenting sessions.

Peer review

This is a very interesting paper that looked into an important problem in the clinical field, taking an original stance. Multiple SEMS, as concluded in the multivariate analysis, seems to be a significant independent risk factor of surgical failure.

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RETROSPECTIVE STUDY

Parallel transjugular intrahepatic portosystemic shunt for controlling portal hypertension complications in cirrhotic patients

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Abstract

AIM: To evaluate the feasibility of a second parallel transjugular intrahepatic portosystemic shunt (TIPS) to reduce portal venous pressure and control complications of portal hypertension.

METHODS: From January 2011 to December 2012, 10 cirrhotic patients were treated for complications of portal hypertension. The demographic data, operative data, postoperative recovery data, hemodynamic data, and complications were analyzed.

RESULTS: Ten patients underwent a primary and parallel TIPS. Technical success rate was 100% with no technical complications. The mean duration of the first operation was 89.20 ± 29.46 min and the second operation was 57.0 ± 12.99 min. The mean portal system pressure decreased from 54.80 ± 4.16 mmHg to 39.0 ± 3.20 mmHg after the primary TIPS and from 44.40 ± 3.95 mmHg to 26.10 ± 4.07 mmHg after the parallel TIPS creation. The mean portosystemic pressure gradient decreased from 43.80 ± 6.18 mmHg to 31.90 ± 2.85 mmHg after the primary TIPS and from 35.60 ± 2.72 mmHg to 15.30 ± 3.27 mmHg after the parallel TIPS creation. Clinical improvement was seen in all patients after the parallel TIPS creation. One patient suffered from transient grade Ⅰ hepatic encephalopathy (HE) after the primary TIPS and four patients experienced transient grade Ⅰ-Ⅱ after the parallel TIPS procedure. Mean hospital stay after the first and second operations were 15.0 ± 3.71 d and 16.90 ± 5.11 d (P = 0.014), respectively. After a mean 14.0 ± 3.13 mo follow-up, ascites and bleeding were well controlled and no stenosis of the stents was found.

CONCLUSION: Parallel TIPS is an effective approach for controlling portal hypertension complications.

Key words: Transjugular intrahepatic portosystemic shunt; Portal hypertension; Portosystemic pressure gradient

Core tip: We retrospectively reviewed 10 patients treated in our institution who underwent a second parallel transjugular intrahepatic portosystemic shunt (TIPS) to reduce portal venous pressure and portosystemic pressure gradient to an acceptable level and control the complications of portal hypertension. We also present our experience evaluating the feasibility and safety of this technique. Parallel TIPS is an effective approach for controlling portal hypertension complications.

He FL, Wang L, Yue ZD, Zhao HW, Liu FQ. Parallel transjugular intrahepatic portosystemic shunt for controlling portal hypertension complications in cirrhotic patients. *World J Gastroenterol* 2014; 20(33): 11835-11839 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i33/11835.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i33.11835

INTRODUCTION

The transjugular intrahepatic portosystemic shunt (TIPS) is an effective way for controlling complications of portal hypertension, including gastrointestinal bleeding and refractory ascites in patients with cirrhosis. TIPS was first described by Rösch *et al*[1] in 1969, and it is currently considered to be one of the most important improvements in interventional therapy and represents a major contribution to treatment of patients with cirrhosis and portal hypertension syndrome[2]. TIPS can reduce portal vein pressure and portosystemic pressure gradient (PSG; pressure difference between portal vein and the cava) in 90%-95% of cases, while a small number of patients do not benefit from TIPS and portal vein pressure remains high after one shunt due to insufficient PSG reduction[3]. Here, we retrospectively reviewed 10 cases out of 205 treated in our institution who underwent a second parallel TIPS to reduce portal venous pressure and PSG to an acceptable level and control the complications of portal hypertension. We also present our experience evaluating the feasibility and safety of this technique.

MATERIALS AND METHODS

Patient data

Between January 2011 and December 2012, 10 patients with cirrhosis (7 males) with a mean age of 52.30 ± 4.52 years underwent a parallel TIPS in our institution (Table 1). All the patients were infected with hepatitis B virus and had complications from cirrhosis and portal hypertension. The indications for TIPS were refractory ascites (n = 9) and gastrointestinal bleeding (n = 1). A second shunt tract was established due to insufficient relief of symptoms and unsuccessful reduction of portal vein pressure. The patients underwent portosystemic shunt twice within 3-6 mo. The hepatic function status evaluated by Child-Pugh classification was class B in eight cases and class C in two. The follow-up time was 9-18 mo and the mean time was 14.0 ± 3.13 mo.

TIPS placement technique

The first TIPS procedure was performed in the Interventional Radiology Suite under local anesthesia. The Rösch-Uchida Transjugular Liver Access Set (Cook, Bloomington, IN, United States) was used for every patient. Right jugular venous access was established with a 10-F sheath. A 5-F multipurpose catheter was used to engage the right hepatic vein and perform angiography, and a curved cannula was then advanced with the guidewire into the right hepatic vein. A sheathed needle was advanced into the portal vein through the liver parenchyma and the guide wire was placed into the portal vein through the sheath. Portal vein angiography was performed with a 5-F pigtail catheter and portal vein pressure and right atrium pressure were measured. Subsequently, the shunt tract was dilated with an angioplasty balloon ranging from 8 to 10 mm, and a covered stent with a diameter of 8 or 10 mm was utilized. If the distal shunt was not sufficient for the shunt tract, an additional stent was utilized to extend the length. The varicose coronary gastric vein was embolized to prevent future gastrointestinal bleeding and the portal vein angiography and pressure measurements were performed again. The PSG was measured before and after the shunt creation.

In nine patients, ascites was not relieved and recurrent gastrointestinal bleeding occurred in the other patient, even though ultrasound proved the patency of all the first shunt tracts. Therefore, a second TIPS was established 3 mo after the first procedure. The right jugular venous access was used for the second shunt tract. Through the first shunt tract, portal vein angiography was carried out (Figure 1A) and pressure was measured. The Rösch-Uchida Transjugular Liver Access Set was used and the

Table 1 Patient data

Characteristic	
Sex (M/F)	7/3
Age (yr, mean ± SD)	52.30 ± 4.52
Child-Pugh classification	
B	8
C	2
Indication for TIPS	
Refractory ascites	9
Gastrointestinal bleeding	1

TIPS: Transjugular intrahepatic portosystemic shunt.

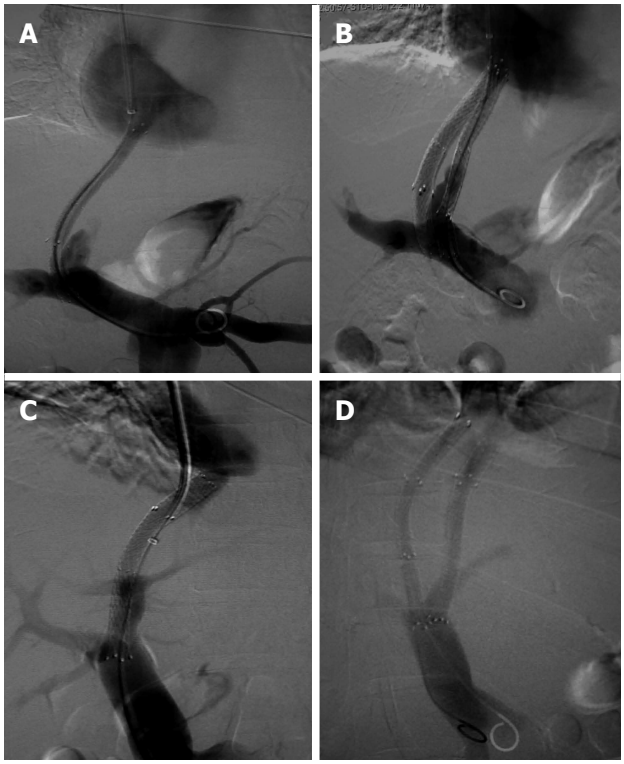

Figure 1 Transjugular intrahepatic portosystemic shunt. A: Angiography showed that the primary transjugular intrahepatic portosystemic shunt (TIPS) tract was patent; B: In the same patient, the parallel TIPS tract was successfully built; C: Parallel shunt tract was directly established from the vena cava to the portal vein; D: In the same patient, the parallel TIPS tract was successfully built.

Table 2 Hemodynamic changes

	Primary TIPS				Parallel TIPS					
	Before (mmHg, mean ± SD)	After (mmHg, mean ± SD)	*t* value	*P* value	Before (mmHg, mean ± SD)	After (mmHg, mean ± SD)	*t* value	*P* value	*t* value[1]	*P* value[1]
Portal venous pressure	54.80 ± 5.16	39.00 ± 3.20	21.73	< 0.001	44.40 ± 3.95	26.10 ± 4.07	11.70	< 0.001	18.35	< 0.001
PSG	43.80 ± 6.18	31.90 ± 2.85	9.38	< 0.001	35.60 ± 2.72	15.30 ± 3.27	67.67	< 0.001	20.01	< 0.001

[1]Comparison of hemodynamic findings before primary and after parallel transjugular intrahepatic portosystemic shunt (TIPS). PSG: Portosystemic pressure gradient.

systemic vein to portal vein parenchymal tract was attempted with the previous technique. With the stents of the first tract as the mark, puncture of the portal vein with the sheathed needle was more targeted and efficient. In four patients, the shunt tract was directly advanced from the vena cava to the portal vein because the right hepatic vein was not available for both tracts. The shunt tract was dilated, the stents were deployed and the varicose coronary gastric vein and other collaterals were embolized as described in this article. Portal vein angiography (Figure 1B) and pressure measurements were once again performed. The PSG was measured as described.

We compared perioperative and postoperative data in all the patients who underwent both TIPS procedures. This study was approved by ethics committee of Shijitan Hospital, Capital Medical University.

Complications and clinical follow-up

All the patients were under close monitoring during the perioperative period. Complications including abdominal cavity hemorrhage, hepatic failure and hepatic encephalopathy (HE) were observed. The patients remained in contact with the doctors who had performed the procedures. Clinical observations included reduction of ascites, gastrointestinal bleeding and HE. They were subjected to ultrasound imaging at 1, 3 and 6 mo after TIPS placement. Evaluations included stent patency, blood flow of the shunt tracts, and presence of ascites.

Statistical analysis

SPSS for Windows version 17.0 was utilized for data processing and statistical analysis, with paired-sample *t* test for data measurement. Data are summarized as frequencies and continuous variables as mean ± standard deviation (SD). $P < 0.05$ was considered statistically significant.

RESULTS

TIPS procedure

All the patients underwent a primary and parallel TIPS procedure, which were successful in all cases, with no technical complications. In six patients the second shunt tract was advanced from the hepatic vein to the portal vein and in the other four patients, the shunt tract was directly advanced from the vena cava to the portal vein (also named direct intrahepatic portacaval shunt; DIPS) (Figure 1C and D). The second tracts were created 3 mo after the first ones. In nine patients, the varicose coronary gastric vein was embolized. The mean duration of the first operation was 89.20 ± 29.46 min and the second operation was 57.0 ± 12.99 min, with a significant difference ($P = 0.001$).

Hemodynamic changes

TIPS altered the portal pressure in all patients after the second operation (Table 2). The mean portal system pressure prior to TIPS placement was 54.80 ± 4.16 mmHg, which decreased to 39.0 ± 3.20 mmHg after the first shunt tract was established ($P < 0.001$). The mean portal system pressure prior to the second TIPS was 44.40 ± 3.95 mmHg and decreased to 26.10 ± 4.07 mmHg after the procedures ($P < 0.001$). The mean PSG prior to the TIPS placement was 43.80 ± 6.18 mmHg, which decreased to 31.90 ± 2.85 mmHg after the first shunt tract was established ($P < 0.001$). The mean PSG prior to the second TIPS was 35.60 ± 2.72 mmHg and decreased to 15.30 ± 3.27 mmHg after the procedures ($P < 0.001$).

Clinical effects

Clinical improvement was seen in all patients after the parallel TIPS procedure. Ascites was not relieved sufficiently after the first procedure, but it disappeared completely in seven patients and decreased obviously in two patients within 7-14 d after the second procedure. Recurrent hemorrhage was not seen in the patient with gastrointestinal bleeding.

Complications

Complications occurred during the procedures and postoperative complications including hepatic failure and HE were observed after the first and second operations. No surgery-related complications such as abdominal cavity hemorrhage or subcutaneous hematoma were found. One patient suffered from transient grade I encephalopathy after the first operation and four patients experienced transient grade I - II HE after the second procedure, which were relieved after medical treatment. One patient with Child-Pugh class C suffered from hepatic failure after the first operation. He and the other Class C patient and one with Child-Pugh class B had hepatic failure after the second operation, which was cured within 1 wk. Two patients suffered from both HE and hepatic failure. The duration of hospital stay for the parallel TIPS procedure was longer than for the primary TIPS procedure: mean hospital stay after the first and second operations was 15.0 ± 3.71 d and 16.90 ± 5.11 d, respectively ($P = 0.014$).

Follow-up

The patients were followed for a mean of 14.0 ± 3.13 mo without loss to follow-up. Two patients with Child-Pugh class C were hospitalized several times due to poor liver function and the other eight patients were followed in the outpatient department. Ascites and bleeding were well controlled during follow-up and no stent stenosis was found by ultrasonic examination. The incidence of transient HE was 30% at 3 mo, 40% at 6 mo and 50% at 12 mo. All patients survived during follow-up. No patient underwent liver transplantation.

DISCUSSION

TIPS is an effective method of treating complications of portal hypertension due to cirrhosis, and has been utilized widely in clinical practice as a safe and minimally invasive procedure[4]. Liver transplantation has not yet been popularized, therefore, TIPS is of major importance worldwide[5]. TIPS has been proven to be effective in 59.4%-91% of cases for secondary prevention of variceal bleeding and in 38%-84% of cases in controlling ascites[6]. The symptoms improved after the parallel TIPS procedure in our study. We believe that uncontrolled ascites and gastrointestinal bleeding after the first TIPS procedure were due to high portal vein pressure and insufficient PSG reduction. In our study, the mean portal vein pressure prior to TIPS was 54.80 ± 4.16 mmHg, which was 10-15 mmHg higher than that in patients who underwent TIPS reported before[6]. The symptoms were controlled after the second procedure when the mean portal vein pressure decreased to 26.10 ± 4.07 mmHg and the mean PSG decreased to 15.30 ± 3.27 mmHg. In 2006, a Brazilian doctor, Néstor[7] reported one case with a second parallel TIPS tract when the first shunt was patent, which reduced the PSG to 9 mmHg and relieved clinical symptoms. Parvinian reported two cases of parallel TIPS for treatment of complications of cirrhotic portal hypertension, with reduction of PSG to 12 and 10 mmHg[8]. The American Association for the Study of Liver Diseases (AASLD) recommends reducing PSG to < 8 mmHg to improve quality of life[9]. Reducing the portal vein pressure and PSG to an acceptable level is the key point of TIPS procedures.

In our study of 10 cases, the technical success rate of a second parallel TIPS tract was 100%. The mean duration of the first procedure was 89.20 ± 29.46 min and the second operation was 57.0 ± 12.99 min, which showed a statistically significant difference. The procedures were all carried out by the same group of doctors, therefore, the reduction in procedure time resulted in more targeted puncture of the portal vein from the vena cava. In TIPS operations, the most critical and time-consuming feature is puncture of the portal vein. In 10%-30% of cases, percutaneous transhepatic portal vein angiography is performed to guide the procedure, and consequently increases the risk of injury to the biliary tract and hepatic vein, and abdominal cavity hemorrhage[10]. In building the parallel tract in this study, with the first tract as the mark, no percutaneous transhepatic portal vein angiography was performed. In four patients, the shunt tract was directly advanced from the vena cava to the portal vein, and the procedures is also named DIPS, due to the absence of the hepatic vein as a shunt outflow. DIPS was first described in 2001 by Petersen *et al*[11]. The hallmark of the DIPS procedure is the use of the caudate lobe as the parenchymal tract to create a side-to-side portocaval shunt. The advantage of the DIPS procedure is not using the hepatic vein as a shunt outflow[12,13]. In a previous study, DIPS was performed by an intravascular ultrasound probe introduced *via* a femoral vein approach[14]. However, no intravascular ultrasound was utilized in our study because the stents of the first tract were a marker for the portal vein. DIPS was the first choice for creating a parallel TIPS tract in cases with an insufficient hepatic vein. In 9 patients the varicose coronary gastric veins were embolized because gastrointestinal bleeding was mainly caused directly by rupture of these vessels in patients with portal hypertension.

Covered stent grafts have been routinely applied in TIPS procedures and have increased the patency rates. Yet, utilization of covered stent grafts in TIPS procedures also increases the risk of HE[15,16]. In our study, 4 of 10 patients experienced transient grade HE, which may have resulted in a significant increase in outflow or large PSG reduction. In cases with a parallel TIPS, close attention should be paid to HE, and treatment against encephalopathy should be given before and after the procedure.

In conclusion, the parallel TIPS procedure is an effective approach for controlling complications of portal hypertension in patients with cirrhosis in whom primary TIPS does not reduce portal vein pressure or PSG to an acceptable level. The parallel TIPS procedure takes less time by using the stents of the first tract as a marker for the portal vein. DIPS is an option for avoiding using the hepatic vein as a shunt outflow. HE is the main complication of the procedure and treatment against encephalopathy should be given before and after the parallel TIPS creation.

COMMENTS

Background

The transjugular intrahepatic portosystemic shunt (TIPS) has been proven to be an effective way for controlling complications of portal hypertension. TIPS can reduce portal vein pressure in 90%-95% of cases, while a small number of patients can benefit from TIPS, and the portal vein pressure remains high after one shunt procedure.

Research frontiers

In 2006, a Brazil doctor, Néstor Hugo Kisilevzky, reported one case with a second parallel TIPS tract when the first shunt was patent, which reduced the portosystemic pressure gradient (PSG) to 9 mmHg and relieved clinical symptoms. Parvinian reported two cases of parallel TIPS for treatment of complications of cirrhotic portal hypertension, reducing the PSG to 12 and 10 mmHg.

Innovations and breakthroughs

This study investigated the demographic data, operative data, postoperative recovery data, hemodynamic data and complications of this new technique in 10 patients.

Applications

The authors presented their experience evaluating the feasibility and safety of this technique. However, randomized controlled trials with more patients should be considered.

Terminology

TIPS is a procedure performed in which a *shunt* is placed between the portal and hepatic veins.

Peer review

In this manuscript, the authors retrospectively analyzed 10 patients who underwent a parallel TIPS in order to reduce the portal venous pressure and PSG to an acceptable level and control complications of portal hypertension between January 2011 and December 2012 at their hospital. Although the results of the manuscript can assist in present their experience evaluating the feasibility and safety of parallel TIPS to reduce the portal venous pressure and PSG to an acceptable level, there remain major concerns.

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RETROSPECTIVE STUDY

Identification of differential proteins in colorectal cancer cells treated with caffeic acid phenethyl ester

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Abstract

AIM: To investigate the molecular mechanisms of the anti-cancer activity of caffeic acid phenethyl ester (CAPE).

METHODS: Protein profiles of human colorectal cancer SW480 cells treated with or without CAPE were analysed using a two-dimensional (2D) electrophoresis gel-based proteomics approach. After electrophoresis, the gels were stained with Coomassie brilliant blue R-250. Digital images were taken with a GS-800 Calibrated Densitometer, and image analysis was performed using PDQuest 2-D Analysis software. The altered proteins following CAPE treatment were further identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry following a database search. The identified proteins were validated by Western blot and immunofluorescence assay.

RESULTS: CAPE induced human colorectal cancer cell apoptosis. Four up-regulated proteins and seven down-regulated proteins in colorectal cancer cells treated with CAPE were found. The identified down-regulated proteins in CAPE-treated colorectal cancer cells were Triosephosphate Isomerase (Tim), Proteasome subunit alpha 4 (PSMA4) protein, Guanine nucleotide binding protein beta, Phosphoserine aminotransferase 1 (PSAT1), PSMA1, Myosin XVIIIB and Tryptophanyl-tRNA synthetase. Notably, CAPE treatment led to the down-regulation of PSAT1 and PSMA1, two proteins that have been implicated in tumorigenesis. The identified up-regulated proteins were Annexin A4, glyceraldehyde-3-phosphate dehydrogenase, Glucosamine-6-phosphate deaminase 1 (GNPDA1), and Glutathione peroxidase (GPX-1). Based on high match scores and potential role in cell growth control, PSMA1, PSAT1, GNPDA1 and GPX-1 were further validated by Western blotting and immunofluorescence assay. PSMA1 and PSAT1 were down-regulated, while GNPDA1 and GPX-1 were up-regulated in CAPE-treated colorectal cancer cells.

CONCLUSION: These differentiated proteins in colorectal cancer cells following CAPE treatment, may be potential molecular targets of CAPE and involved in the anti-cancer effect of CAPE.

Key words: Caffeic acid phenethyl ester; Colorectal cancer; Proteomics; Two-dimensional electrophoresis; Mass spectrometry

Core tip: To investigate the molecular mechanisms of the anti-cancer activity of caffeic acid phenethyl ester (CAPE), CAPE-treated colorectal cancer SW480 cells were analysed by a 2D-gel based proteomics approach. Four up-regulated proteins and seven down-regulated proteins in CAPE-treated SW480 cells were found and further identified by matrix-assisted laser desorption/

ionization time-of-flight mass spectrometry following a database search. The down-regulated proteins, PSMA1 and PSAT1 and up-regulated proteins GNPDA1 and GPX-1 were validated by Western blotting. The two tumorigenesis associated proteins, PSMA1 and PSAT1, were further confirmed by immunofluorescence assay. These differentiated proteins in colorectal cancer cells following CAPE treatment, may be potential molecular targets of CAPE and involved in the anti-cancer effect of CAPE.

He YJ, Li WL, Liu BH, Dong H, Mou ZR, Wu YZ. Identification of differential proteins in colorectal cancer cells treated with caffeic acid phenethyl ester. *World J Gastroenterol* 2014; 20(33): 11840-11849 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i33/11840.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i33.11840

INTRODUCTION

Colorectal cancer (CRC) is one of the most commonly diagnosed malignancies and the third deadliest cancer in humans. In 2012, it was estimated that 143460 people in the United States had been diagnosed with colorectal cancer and that 51690 will die from this disease[1,2]. In the last few decades, enormous advances in the diagnosis and treatment of CRC have been made, and molecular biology has clarified some of the mechanisms involved in the carcinogenic process. However, patient prognosis is still poor; after curative resections, approximately 50% of patients succumb to recurrent and metastatic disease during the first 2 years of follow-up. For this reason, novel anti-cancer drugs for CRC are urgently needed.

Caffeic acid phenethyl ester (CAPE), a component of propolis is a phenolic antioxidant. CAPE has been shown to help in host defence through its anti-viral and anti-bacterial activity. In addition, the immunoregulatory properties, anti-inflammatory activity and anti-cancer activity of CAPE have been reported. Several studies have demonstrated that CAPE has anti-proliferative effects by inducing apoptosis in various tumour cells *in vitro*[3-7] and *in vivo*[8,9]. CAPE also inhibits the development of azoxymethane-induced aberrant crypts in the colon of rats[10].

Multiple molecular mechanisms seem to be involved in the anti-cancer effects of CAPE. We have previously shown that decreased β-catenin and associated signalling pathways may mediate the anti-cancer effects of CAPE[2]. It has been reported that CAPE inhibits tumor necrosis factor alpha-dependent nuclear factor kappa beta (NFκB) activation *via* direct inhibitory protein kappaB kinase inhibition and Nuclear factor-erythroid 2 p45 (NF-E2)-related factor 2 pathway activation[11]. Previous studies have also shown that Mcl-1 down-regulation, Bcl-2 expression, and Bax up-regulation, as well as activation of caspase-8, caspase-3, and PARP, are associated with CAPE-dependent cellular apoptosis[12,13]. However, the exact anti-tumour mechanism of CAPE is not fully understood.

To understand the mechanism of the anti-cancer activity of CAPE, CAPE-treated colorectal cancer SW480 cells were analysed by a 2D-gel based proteomics approach. Differentially expressed proteins were identified by mass spectrometry and then validated by Western blotting and confocal microscopy.

MATERIALS AND METHODS

Cell culture

The human CRC cell line SW480 was purchased from the American Type Culture Collection. The cells were cultured in RPMI-1640 medium supplemented with penicillin G (100 U/mL), streptomycin (100 μg/mL), and 10% foetal calf serum. The cells were grown at 37 ℃ in a humidified atmosphere of 5% CO_2 and were routinely sub-cultured using 0.25% (w/v) trypsin-EDTA solution. All cell culture reagents were purchased from GIBCO (Carlsbad, United States). For CAPE treatment, CAPE was dissolved in DMSO and adjusted to a working concentration with culture medium before use (DMSO concentration was 0.1%). CAPE was added to the culture medium on the second day at a working concentration of 10 μg/mL.

TUNEL staining

SW480 cells were grown on poly-L-lysine coated slides in a six-well plate. After treatment with or without CAPE for 48 h, the slides were gently washed three times in 0.1 mol/L PBS (pH = 7.4) and fixed with 4% paraformaldehyde. To determine cellular apoptosis, terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assays were performed using the TUNEL Detection kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. All samples were observed under a microscope. Cell apoptosis was determined by counting TUNEL positive cells under a light microscope at × 40 objective.

Protein separation by 2-D electrophoresis

SW480 cells were cultured in RPMI 1640 medium with or without CAPE (10 μg/mL) for 48 h. The cells were carefully collected using a cell scraper. All reagents for 2D electrophoresis were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden), except those otherwise indicated. To perform 2D electrophoresis, SW480 tumour cells were suspended in lysis buffer containing 40 mmol/L Tris, 8 mol/L urea, 4% CHAPS, 60 mmol/L DTT, 0.8% IPG buffer (pH = 3-10), and protease inhibitor cocktail (Roche, Mannheim, Germany). Protein concentration was measured with the DC Protein Assay (BioRad, United States). Proteins (500 mg/gel) were loaded into IEF gels (pH = 3-10). The gels were immersed overnight in hydration buffer containing 8 M urea, 4% CHAPS, 60 mmol/L DTT, and 0.5% IPG buffer. After sample loading, IEF gels were run at 200 V for 1 h, 500 V for 1 h, 1000 V for 1 h and then were gradually increased to 8000 V for 5-6 h. Focusing was carried out at 35000 V·h. After

IEF, IPG strips were equilibrated twice in equilibration buffer (50 mmol/L Tris-HCl (pH = 8.8), 6 mol/L urea, 30% glycerol, 2% SDS). In the first equilibration, 100 mg of DTT was dissolved in 10 mL of equilibration buffer, and 400 mg of iodoacetamide was added in the second equilibration. The strips were then transferred onto vertical slab 12.5% SDS-PAGE gels and sealed with 0.5% low melting point agarose.

Image analysis

After electrophoresis, the gels were stained with Coomassie brilliant blue R-250. Digital images were taken with a GS-800 Calibrated Densitometer (BioRad, USA), and image analysis was performed with PDQuest 2-D Analysis software (BioRad, United States).

Protein in-gel enzyme digestion and identification

In-gel digestion was performed as described by Rosenfeld[14]. Briefly, spots were excised from the stained gel, destained with 25 mmol/L ammonium bicarbonate/50% acetonitrile (ACN), and then dried with a SpeedVac plus SC1 10 (Savant Holbook, United States). The dried gels were rehydrated in trypsin solution (Promega, United States) at 37 ℃ overnight. After rehydration, peptides were first eluted with 5% trifluoroacetic acid (TFA) at 40 ℃ for 1 h, and then eluted with 2.5% TFA/50% ACN at 30 ℃ for 1 h. ACN was removed by centrifugation in a vacuum centrifuge. The peptides were concentrated using C18 pipette tips (Millipore, Bedford, MA, United States). Analysis was performed primarily using the matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometer (Bruker, Germany). Peptide mixtures were analysed using a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma, United States) in acetone containing 1% TFA. Peptides were selected in the mass range of 800-4000 Da. The peptide sequence was determined with MASCOT software. Sequence homology was analysed using the MASCOT program and the NCBI BLAST online search service. The database NCBInr 20060731 was used.

Western blotting

SW480 cells were treated with or without CAPE (10 μg/mL) for 48 h. The cells were lysed in SDS-sample loading buffer and boiled at 100 ℃ for 5 min. The cell lysates were then subjected to SDS-PAGE. Proteins in the gel were then transferred onto PVDF membranes. The PVDF membranes were incubated for 2 h in blocking buffer (5% milk in 10 mmol/L Tris-HCl (pH = 7.5), 2.5 mmol/L EDTA (pH = 8.0), 50 mmol/L NaCl). The membranes were then incubated in antibodies against PSMA1, PSAT1, GNPDA1 or GPX1 (Sigma-Aldrich, United States) at a dilution of 1:1000 for 2 h at room temperature. After washing three times with washing buffer (TBS buffer containing 0.01% Tween 20), the membranes were incubated with HRP-conjugated anti-human IgG antibodies (Zhongshan Inc., Beijing, China) at a dilution of 1:5000 for 1 h at room temperature. Immunodetection was determined using the ECL-plus kit (Roche, United States) and autoradiography.

Immunofluorescence assay

SW480 cells grown on glass coverslips were treated with or without CAPE (10 μg/mL) for 48 h under standard culture conditions as described above. The cells were washed with PBS and fixed with methanol for 20 min. Incubation with anti-PSMA1, and anti-PSAT1 monoclonal antibody (1:500) was carried out overnight at 4 ℃. This step was followed by incubation with FITC-conjugated secondary antibody (1:1000) for 1 h at room temperature. DAPI was used to stain the nucleus. Images were captured using a laser scanning confocal microscope (Leica, Germany).

RESULTS

CAPE inhibits tumour cell growth and induces apoptosis

To set up the cell culture system with CAPE treatment, SW480 cells were treated with CAPE at 5 μg/mL or 10 μg/mL, and cell growth was monitored daily by cell counting for a few days. Similar to a previous study[2], 10 μg/mL of CAPE effectively inhibited cell growth when compared to untreated control cells (data not shown). To determine if cell growth inhibition was caused by cell apoptosis, TUNEL assay was performed. We found a dose-dependent increase in cell apoptosis following treatment with CAPE (Figure 1A-D). Our data suggest that the growth inhibitory effect of CAPE may be associated with an increase in cell apoptosis.

Identification of differentially expressed proteins by the proteomics approach

To investigate the molecular mechanisms of the anticancer activity of CAPE, CAPE-treated colorectal cancer SW480 cells were analysed by a 2D-gel based proteomics approach. We used a cell viability assay to determine the optimum CAPE concentration of 10 μg/mL for cell treatment over 48 h.

Protein expression profiles in SW480 cells with or without CAPE treatment were compared by 2D electrophoresis (2-DE). Approximately 250 protein spots in untreated (Figure 2A) and treated cells (Figure 2B) were detected on the Coomassie stained gels. All spots were matched by gel-to-gel comparison using PDQuest software, and the difference in the relative abundance of each protein spot was analysed. Four up-regulated and seven down-regulated protein spots in the treated SW480 cells were found repeatedly (Figure 2C). Those eleven highly repeatable proteins were excised and then identified by MALDI-TOF mass spectrometry and a database search. The seven down-regulated proteins in response to CAPE treatment were Triosephosphate isomerase, PSMA4 protein, guanine nucleotide-binding protein, PSAT1, PSMA1, myosin WVIIIB, and human tryptophanal-tRNA synthetase (Table 1). The up-regulated proteins were Annexin A4, glyceraldehyde-3-phosphate dehydrogenase,

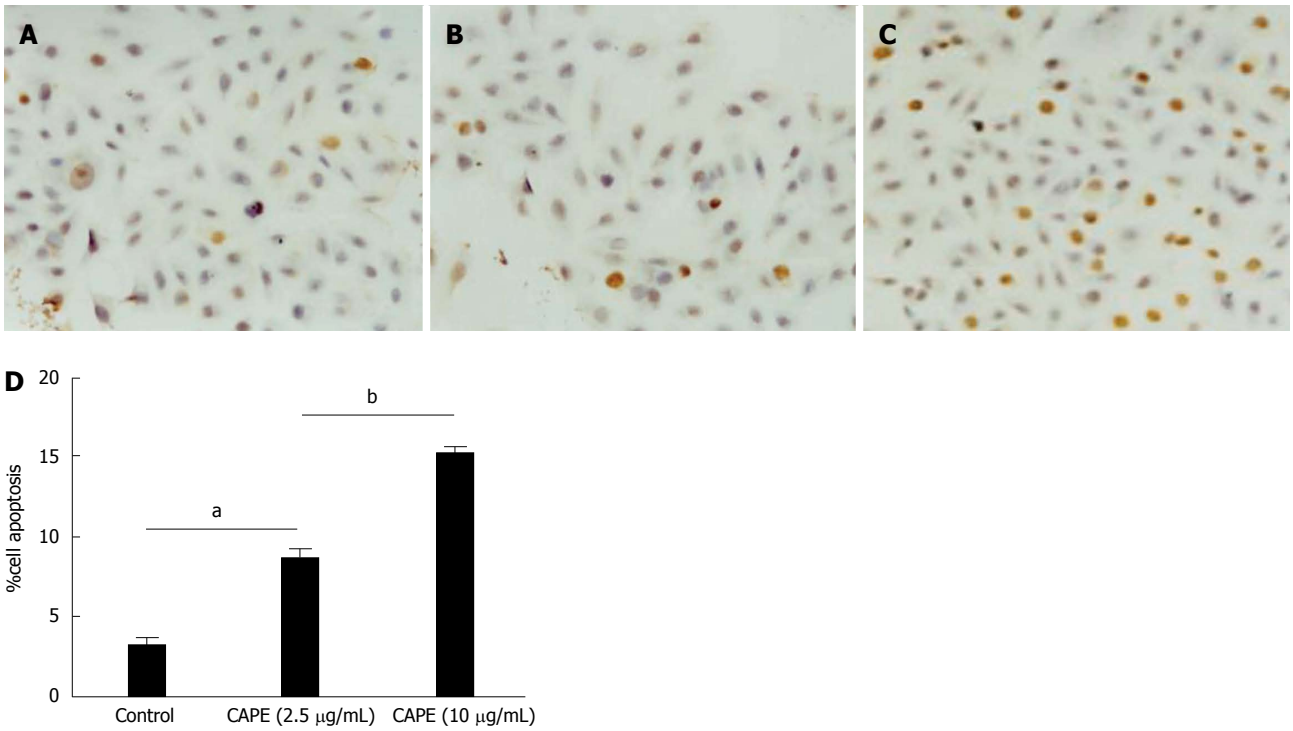

Figure 1 Caffeic acid phenethyl ester induces colorectal cancer cell apoptosis. SW480 cells were treated with caffeic acid phenethyl ester (CAPE) in 24-well plates. For TUNEL staining, cells were treated without (A), with 2.5 μg/mL (B), or 10 μg/mL (C) CAPE for 48 h and cell apoptosis was examined using the TUNEL detection kit. Cell apoptosis was determined by counting TUNEL positive cells under a light microscope at × 40 objective (D). Results are representative of 3 independent experiments with similar results. $^{a}P < 0.05$ *vs* controls; $^{b}P < 0.01$, CAPE (2.5 μg/mL) *vs* CAPE (10 μg/mL).

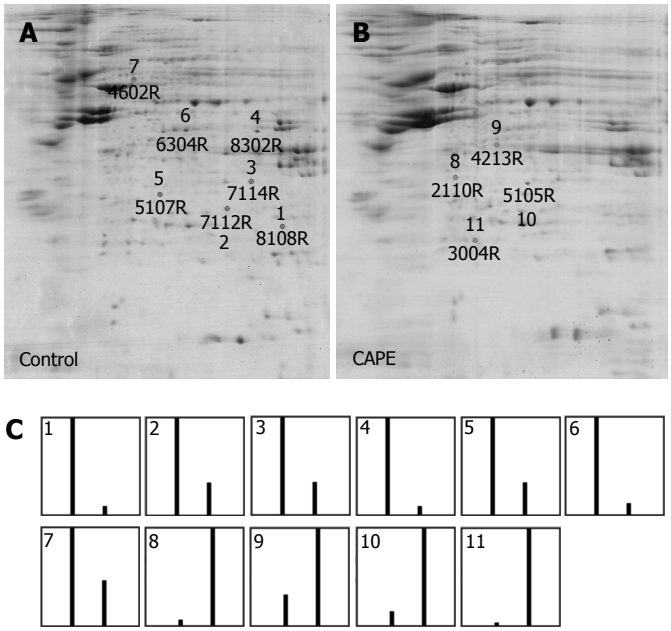

Figure 2 Protein profiles of SW480 cells treated with caffeic acid phenethyl ester by two-dimensional PAGE. SW480 cells were treated without (A) or with (B) 10 μg/mL caffeic acid phenethyl ester (CAPE) for 48 h and then harvested for 2D-PAGE analysis. 500 mg of cellular protein was applied to the IEF gel. After electrophoresis, the gels were stained with Coomassie brilliant blue R-250 and the proteins were analysed by PDQuest 2-D analysis software. The marked spots shown on the gels were the down-regulated proteins (shown by numbers 1-7) and the up-regulated proteins (shown by numbers 8-11). The relative abundance of each differentially expressed protein in the 2 gels was analysed by PDQuest (C). The first bar shown in the graph is the relative abundance of the proteins in untreated cells and the second bar is the relative abundance of the proteins in CAPE-treated cells. Results are representative of 3 independent experiments with similar results.

Glucosamine-6-phosphate deaminase 1 (GNPDA1), and glutathione peroxidase (GPX-1) (Table 2).

Validation of differentially expressed proteins

To validate the above proteomic findings, the expression levels of some identified proteins were examined by Western blot analysis. Proteins were selected for further analysis based on both their high match score and their probable role in cell growth control. Similar to our earlier observation (Figure 2), the expression of PSMA1 and PSAT1 was down-regulated, and the expression of GNPDA1 and GPX-1 was up-regulated in CAPE-treated cells (Figure 3A and B). The identity of the two tumorigenesis associated proteins, PSMA1 and PSAT1, were further confirmed by immunofluorescence assay. PSMA1 and PSAT1 were mainly expressed on the cell membrane and

Table 1 Identification of down-regulated proteins in SW480 cells treated with caffeic acid phenethyl ester

No.	Protein name	Accession No.	Matched peptides	Protein sequence coverage	Mascot score	MW/pI
1	Triosephosphate Isomerase (Tim)	gi|15079533	FFVGGNWK KQSLGELIGTLNAAK VPADTEVVCAPPTAYIDFAR IAVAAQNCYK VTNGAFTGEISPGMIK DCGATWVVLGHSER RHVFGESDELIGQK HVFGESDELIGQK VAHALAEGLGVIACIGEK VVLAYEPVWAIGTGK TATPQQAQEVHEK SNVSDAVAQSTR IIYGGSVTGATCK ELASQPDVDGFLVGGASLKPEFVDIINAK	79%	131	26.8/6.51
2	PSMA4 protein	gi|34783332	TTIFSPEGR LLDEVFFSEK LNEDMACSVAGITSDANVLTNELR YLLQYQEPIPCEQLVTALCDIK RPFGVSLLYIGWDK HYGFQLYQSDPSGNYGGWK ATCIGNNSAAAVSMLK QKEVEQLIK KHEEEEAK	50%	70	29.6/7.56
3	Guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1	gi|5174447	GHNGWVTQIATTPQFPDMILSASR DETNYGIPQR GHSHFVSDVVISSDGQFALSGSWDGTLR LWDLTTGTTTR DVLSVAFSSDNR YTVQDESHSEWVSCVR FSPNSSNPIIVSCGWDK FSPNSSNPIIVSCGWDKLVK VWNLANCK TNHIGHTGYLNTVTVSPDGSLCASGGK DGQAMLWDLNEGK HLYTLDGGDIINALCFSPNR YWLCAATGPSIK IIVDELKQEVISTSSK AEPPQCTSLAWSADGQTLFAGYTDNLVR VWQVTIGTR	80%	154	35.5/7.6
4	Phosphoserine aminotransferase 1 (PSAT1)	gi|17390289	QVVNFGPGPAK LPHSVLLEIQK GVGISVLEMSHR CADYVVTGAWSAK FGTINIVHPK FGTINIVHPKLGSYTK GAVLVCDMSSNFLSKPVDVSK NVGSAGVTVVIVR DDLLGFALR SQTIYEIIDNSQGFYVCPVEPQNR ASLYNAVTIEDVQK	38%	79	40.9/7.56
5	Proteasome (prosome, macropain) subunit, alpha type, 1 (PSMA1)	gi|18490859	NQYDNDVTVWSPQGR IHQIEYAMEAVK IHQIEYAMEAVKQGSATVGLK ILHVDNHIGISIAGLTADAR LLCNFMR FVFDRPLPVSR HMSEFMECNLNELVK DLEFTIYDDDDVSPFLEGLEERPQR AQPAQPADEPAEKADEPMEH	50%	81	29.8/6.15

6	Myosin XVIIIB	gi|51317366	DRQGTRPQAQGPGEGVRPGK EGAEPTNTVEKGNVSK STTGKAGESWDK MGQPQGKSGNAGEAR AGDGAGALETELEGPSQPALEK AGDGAGALETELEGPSQPALEKDAERPR RDQSIVALGWSGAGK QKAAAAFAQLQGAMEMLGISESEQR AAAAFAQLQGAMEMLGISESEQRAVWR QIIQQMTFGPSR+ Oxidation (M) SFSSHHLSMASIMVVDSPGFQNPR + 2 Oxidation (M);Pyro-glu (N-term Q) LQLLFYQRTFVSTLQR AVAGLEGTSQQALQR LQMDALTSMIK+ 2 Oxidation (M) NPTGGADEWQMR + 2 Oxidation (M) FDLQLAQALGESVFEK WELGQLQQQLKQK FELEIERMK+ Oxidation (M) RTHALLSDVQLLLGTMEDGK HKLQEQLQVAQMR+ Oxidation (M) DSLIKMGEELSQAATSESQQR+ Oxidation (M) CMELEKYVEELAAVR + Oxidation (M) INEEAGDTERTQSALALSR + Oxidation (M) DMLLSPTLRPR+ Oxidation (M) DMLLSPTLRPRR	14%	75	28.7/6.45
7	Chain B, A Short Peptide Insertion Crucial For Angiostatic Activity Of Human Tryptophanyl-tRNA Synthetase	gi|42543731	EDFVDPWTVQTSSAKGIDYDK ATGQRPHHFLR GIFFSHR GIFFSHRDMNQVLDAYENK KPFYLYTGR GPSSEAMHVGHLIPFIFTK WLQDVFNVPLVIQMTDDEK+ Oxidation (M) TFIFSDLDYMGMSSGFYK + Oxidation (M) HVTFNQVK GIFGFTDSDCIGK ISFPAIQAAPSFSNSFPQIFR IGYPKPALLHSTFFPALQGAQTK MSASDPNSSIFLTDTAK ALIEVLQPLIAEHQAR KLSFDFQ	56%	79	44.9/6.41

in the cytosol (Figure 3B and C). After CAPE treatment, the expression levels of PSMA1 and PSAT1 were altered, although the cellular localisation of these proteins did not change (Figure 3B and C).

DISCUSSION

Propolis has been used in folk medicine since ancient times and has been noted to exhibit immunoregulatory, anti-bacterial, anti-inflammatory, and anti-tumorigenic activities in different models[15-17]. CAPE is a component of propolis and is therefore implicated in the activity of propolis. CAPE has been shown to selectively target tumour cells and to inhibit tumour cell proliferation. In addition, CAPE has been demonstrated to induce apoptosis in different types of tumours including breast cancer[18], myeloid leukaemia[13], cervical cancer[12], hepatocarcinoma cell[19], cholangiocarcinoma[7], and glioma[20].

In our previous studies, we demonstrated that CAPE could inhibit colorectal cancer cell proliferation by inducing cell cycle arrest and apoptosis[2]. Recently, it was shown that CAPE was a specific inhibitor of nuclear factor κB, inducing apoptosis *via* activation of the Fas signalling pathway in human tumour cells[21]. Other signalling pathways may also be involved[2,11,13,19]. To investigate the molecular mechanisms of the anti-cancer activity of CAPE, we compared the protein expression profiles of treated SW480 cells using 2D electrophoresis. Highly repeatable protein spots were selected and identified by MALDI-TOF mass spectrometry and online database searching.

PSAT1 belongs to subgroup Ⅳ of the aminotransferases and plays a crucial role in linking the central catabolic pathways (glycolysis) and amino acid biosynthesis pathways. PSAT1 catalyses the second step in the biosynthesis of the amino acid, serine, which in turn, is the crucial carbon source for purine nucleotides, phosphatidylcholine, phosphatidylserine, and other cellular metabolites. PSAT1 is weakly expressed in the normal colon, but overexpressed in colon cancer with increased expression as disease progresses[22,23]. PSAT1 expression was shown to be up-regulated during the colorectal adenoma-to-carcinoma sequence by proteomic technology[24]. Recently, it has been reported that the overexpression of PSAT1 stimulates cell growth and increases the chemoresistance of colon cancer cells[25], indicating that overexpression of

Table 2 Identification of up-regulated proteins in SW480 cells treated with caffeic acid phenethyl ester

No.	Protein name	Accession No.	Matched peptides	Protein sequence coverage	Mascot score	MW/pI
8	Annexin A4	gi|12652859	AASGFNAMEDAQTLR GLGTDEDAIISVLAYR GAGTDEGCLIEILASR ISQTYQQQYGR SLEDDIRSDTSFMFQR + Oxidation (M) SDTSFMFQR+ Oxidation (M) VLVSLSAGGR DEGNYLDDALVR QDAQDLYEAGEK FLTVLCSR NRNHLLHVFDEYK NHLLHVFDEYK NHLLHVFDEYKR SETSGSFEDALLAIVK NKSAYFAEK GLGTDDNTLIR VMVSRAEIDMLDIR+2 Oxidation (M) AEIDMLDIR	56%	105	36.2/5.65
9	Glyceraldehyde-3-phosphate dehydrogenase	gi|31645	LVINGNPITIFQERDPSK WGDAGAEYVVESTGVFTTMEK RVIISAPSADAPMFVMGVNHEK IISNASCTTNCLAPLAK VIHDNFGIVEGLMTTVHAITATQK GALQNIIPASTGAAK VPTANVSVVDLTCR LISWYDNEFGYSNR VVDLMAHMASK+2 Oxidation (M)	46%	76	36.2/8.28
10	Glucosamine-6-phosphate deaminase 1 (GNPDA1)	gi|18490843	IIQFNPGPEK YFTLGLPTGSTPLGCYK TFNMDEYVGLPR AAGGIELFVGGIGPDGHIAFNEPGSSLVSR TLAMDTILANAR VPTMALTVGVGTVMDAR EVMILITGAHKAFALYK AIEEGVNHMWTVSAFQQHPR TVFVCDEDATLELK ETEKSQSSK	54%	90	32.8/6.42
11	Glutathione peroxidase 1 (GPX1)	gi|14717805	GLVVLGFPCNQFGHQENAK YVRPGGGFEPNFMLFEK CEVNGAGAHPLFAFLR EALPAPSDDATALMTDPKLITWSPVCR LITWSPVCR FLVGPDGVPLR FLVGPDGVPLRR RFQTIDIEPDIEALLSQGPSCA	56%	70	22.2/6.15

PSAT1 may be involved in tumorigenesis and promotes cell growth. In contrast, down-regulation of PAST1 in CAPE-treated colorectal cancer cells may be associated with cell growth inhibition.

Proteasomes are distributed throughout eukaryotic cells at a high concentration and cleave peptides in an ATP/ubiquitin-dependent process in non-lysosomal pathways. PSMA1 is a subunit that is strategically located at the mouth of the core of the proteasome barrel. While PSMA1 is not part of the catalytic machinery of the proteasome, it likely plays a role in gating the entry of proteins into the barrel. PSMA1 has been shown to bind specifically with Notch 3 protein in a yeast two-hybrid assay, which results in the inhibition of proteasome activity[26]. PSMA1 has been reported to be over-expressed in breast cancer tissue compared to adjacent normal tissue[27], suggesting that PSMA1 may be involved in tumorigenesis. Similar to PSAT1, PSMA1 was down-regulated in CAPE-treated CRC cells, suggesting that PSMA1 is not only an important regulator of biological processes, but also involved in the anti-cancer activity of CAPE.

GNPDA or glucosamine-6-phosphate isomerase (GNPI) is an allosteric enzyme that catalyses the reversible conversion of D-glucosamine-6-phosphate into D-fructose-6-phosphate and ammonium[28]. Although GNPI has been found to be expressed in human tissues and some cancer cell lines ubiquitously[29], its role in tumorigenesis and the anti-cancer effect of CAPE is unknown. However, lower expression of another up-regulated protein, GPX1, a selenium-containing antioxidant enzyme, is associated with aggressiveness and poor

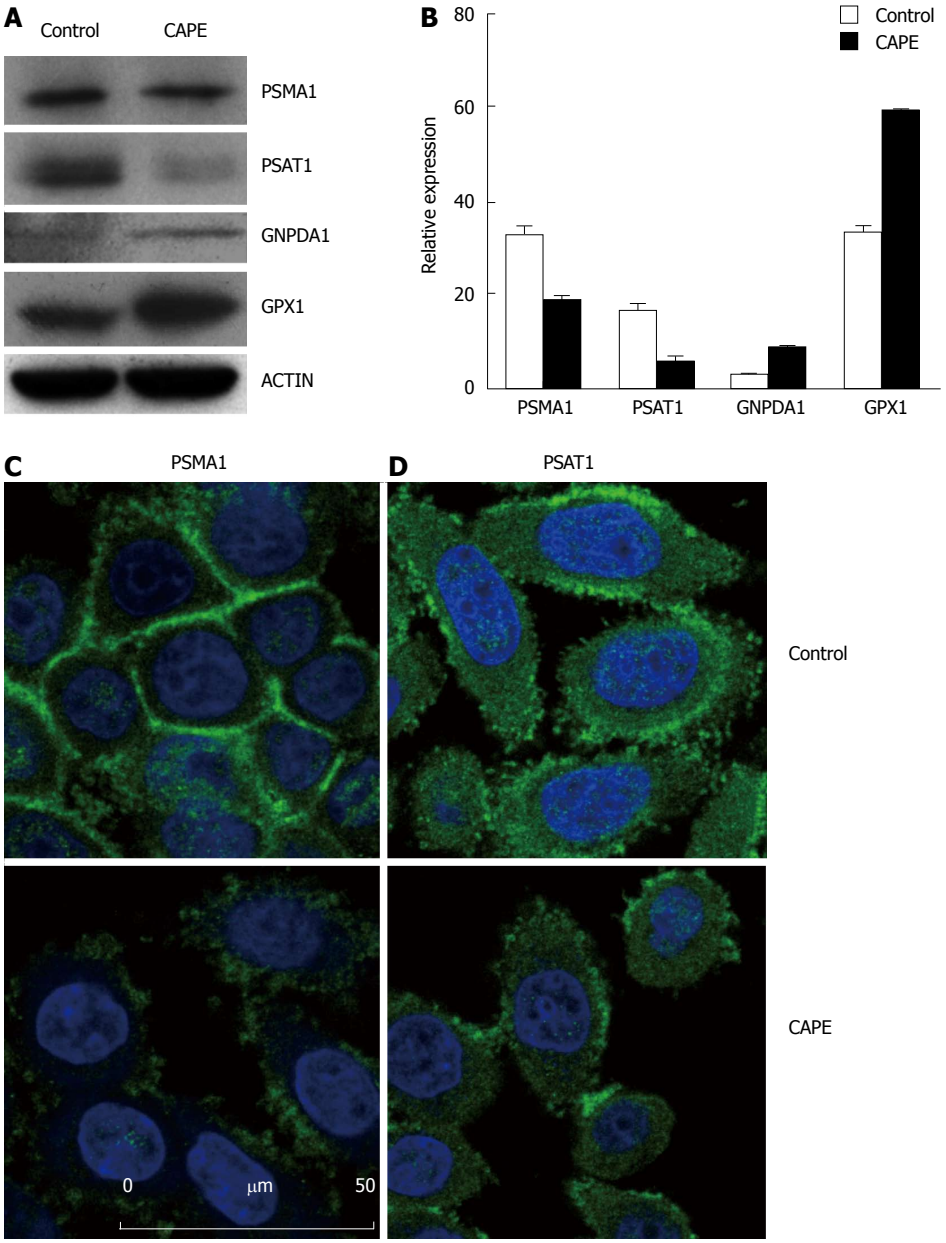

Figure 3 Validation of differentially expressed proteins in SW480 treated with caffeic acid phenethyl ester. SW480 cells were treated without (control) and with 10 μg/mL CAPE for 48 h and then harvested for Western blotting (A and B) or immunofluorescence assay (C and D). For Western blotting, beta-actin was included as the internal control. Densitometric analysis was performed and the integrated density values are presented as the ratio of each protein over the beta-actin protein (B). For the immunofluorescence assay, SW480 cells were grown on glass coverslips and treated without (control) or with CAPE (10 μg/mL) for 48 h. The cells were washed with PBS and fixed with methanol. Anti-PSMA1 (A), and anti-PSAT1 (B) monoclonal antibodies were applied as the primary antibodies, and then, FITC-conjugated secondary antibodies were used. DAPI was used to stain the nucleus. Results are representative of 2 independent experiments with similar results. PSMA1: Proteasome subunit alpha 1; PSAT1: Phosphoserine aminotransferase 1.

survival in patients with cancer[30-32]. GPX-1 may have cancer-supressing effects and up-regulation of GPX1 in CAPE-treated colorectal cancer cells might also be associated with the anti-cancer effect of CAPE.

In conclusion, we found that CAPE induced cell apoptosis and a differential protein expression profile. In particular, CAPE treatment resulted in down-regulation of proteins previously implicated in tumorigenesis. Down-regulated PSAT1 and PSMA1 and up-regulated GPX-1 in CAPE treated-colorectal cancer cells may be potential molecular targets of CAPE and involved in the anti-cancer effect of CAPE.

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COMMENTS

Background

Colorectal cancer is one of the most commonly diagnosed malignancies and the third deadliest cancer in humans. Caffeic acid phenethyl ester (CAPE) is a phenolic antioxidant, which is known to suppress the growth of tumor cells and induce cell apoptosis. However, the molecular mechanisms of the anti-cancer

activity of CAPE are unclear.

Research frontiers

CAPE is an active component of propolis and has various biological and pharmacological functions including immunoregulatory, anti-inflammatory, anti-viral, anti-bacterial, and anti-cancer activities. Several studies have demonstrated that CAPE has anti-proliferative effects by inducing apoptosis in various tumour cells *in vitro* and *in vivo*. CAPE also inhibits the development of azoxymethane-induced aberrant crypts in the colon of rats.

Innovations and breakthroughs

Based on a proteomic approach, several altered proteins were identified in CAPE-treated human colorectal cancer cells. Phosphoserine aminotransferase 1 (PSAT1) and Proteasome subunit alpha 1 (PSMA1), have been shown to be overexpressed in human cancer tissues, while low expression of Glutathione peroxidase (GPX-1) is known to be associated with aggressiveness and poor survival in patients with cancer. Down-regulated PSAT1 and PSMA1 and up-regulated GPX-1 in CAPE treated-colorectal cancer cells may be potential molecular targets of CAPE and involved in the anti-cancer effect of CAPE.

Applications

These findings suggest that CAPE mediates its anti-cancer effect by regulating the expression of important molecules.

Terminology

Proteomics is the study of the structure and function of proteins in a cell or tissue at a specific time under certain pre-defined conditions. CAPE is a natural phenolic chemical compound. It is found in a variety of plants and is also a component of propolis found in honeybee hives.

Peer review

The authors of this paper studied the mechanism involved in the inhibition by CAPE of colorectal cancer cells, and identified differential protein expression with or without CAPE-treatment. They concluded that CAPE-treatment down-regulated 7 proteins including PSAT1 and PSMA1 that played important roles in tumorigenesis but up-regulated 4 proteins including GNPDA1 and GPX1. The article is well written. Experimental design was logically followed through. Method and results were well presented.

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OBSERVATIONAL STUDY

Radiologic-pathologic correlation of three-dimensional shear-wave elastographic findings in assessing the liver ablation volume after radiofrequency ablation

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Abstract

AIM: To evaluate the usefulness of three-dimensional (3D) shear-wave elastography (SWE) in assessing the liver ablation volume after radiofrequency (RF) ablation.

METHODS: RF ablation was performed *in vivo* in 10 rat livers using a 15-gauge expandable RF needle. 3D SWE as well as B-mode ultrasound (US) were performed 15 min after ablation. The acquired 3D volume data were rendered as multislice images (interslice distance: 1.10 mm), and the estimated ablation volumes were calculated. The 3D SWE findings were compared against digitized photographs of gross pathological and histopathological specimens of the livers obtained in the same sectional planes as the 3D SWE multislice images. The ablation volumes were also estimated by gross pathological examination of the livers, and the results were then compared with those obtained by 3D SWE.

RESULTS: In B-mode US images, the ablation zone appeared as a hypoechoic area with a peripheral hyperechoic rim; however, the findings were too indistinct to be useful for estimating the ablation area. 3D SWE depicted the ablation area and volume more clearly. In the images showing the largest ablation area, the mean kPa values of the peripheral rim, central zone, and non-ablated zone were 13.1 ± 1.5 kPa, 59.1 ± 21.9 kPa, and 4.3 ± 0.8 kPa, respectively. The ablation volumes depicted by 3D SWE correlated well with those estimated from gross pathological examination (r^2 = 0.9305, P = 0.00001). The congestion and diapedesis of red blood cells observed in histopathological examination were greater in the peripheral rim of the ablation zone than in the central zone.

CONCLUSION: 3D SWE outperforms B-mode US in delineating ablated areas in the liver and is therefore more reliable for spatially delineating thermal lesions created by RF ablation.

Key words: Radiofrequency ablation; Liver; Ultrasound; Shear-wave elastography; Three-dimensional

Core tip: Three-dimensional shear-wave elastography is a reliable noninvasive technique that may be useful for the real-time assessment of treatment efficacy immediately after radiofrequency ablation procedures. It is superior to B-mode ultrasound in delineating the ablated areas in the liver. The threshold value for determining remaining cell viability was found to be 13.1 ± 1.5 kPa.

Sugimoto K, Oshiro H, Ogawa S, Honjo M, Hara T, Moriyasu F. Radiologic-pathologic correlation of three-dimensional shear-

wave elastographic findings in assessing the liver ablation volume after radiofrequency ablation. *World J Gastroenterol* 2014; 20(33): 11850-11855 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i33/11850.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i33.11850

INTRODUCTION

Radiofrequency (RF) ablation has recently been reported to be useful for the treatment of malignant liver tumors[1,2]. Because of its ease of use, widespread availability, potential for repeated use in solitary or multifocal hepatic disease, and low cost compared with laparoscopic and open surgical approaches, the range of applications of RF ablation has been expanding to include the treatment of malignancies in a variety of organs.

On the other hand, a notable disadvantage of RF ablation is its lower success rate for complete local tumor eradication compared with more invasive surgical methods[3]. The ability to perform evaluation during or immediately after ablation procedures would therefore be very helpful, making it possible to determine the completeness of ablation in real time and to perform further intervention if remaining viable tumor is found.

In recent decades, there has been an increasing interest in assessing the viscoelastic properties of tissues using ultrasound (US) elastography. Among the various techniques employed in US elastography, shear-wave elastography (SWE) is a highly reproducible method for measuring the propagation speed of shear waves within tissues to locally quantify tissue stiffness in kilopascals (kPa) or meters per second[4]. Three-dimensional (3D) SWE has also recently been developed. This method can provide 3D color-coded elasticity maps of tissue stiffness and quantitative 3D elastography volume images in a single acquisition[5].

Moreover, elastography has recently been shown to be useful for monitoring the effects of ablative therapies on tumors. Early investigations at other laboratories as well as a number of clinical studies suggest that US elastography may be superior to conventional US for monitoring the lesions created by RF ablation[6]. If this is found to be the case, US elastography may prove to be a more useful method for precisely assessing ablated areas. As additional advantages, the administration of contrast agent is not required and real-time observation is possible.

The goal of the present study was to investigate in detail how closely the boundaries of thermal lesions observed by 3D SWE correspond to the actual boundaries of tissue ablation determined by histopathological examination. Specifically, we first determined a threshold kPa value that is correlated with the presence of viable cells indentified in histopathological specimens. Next, based on this threshold value, we compared the volumes of the thermal lesions depicted by 3D SWE with those estimated by gross pathological examination.

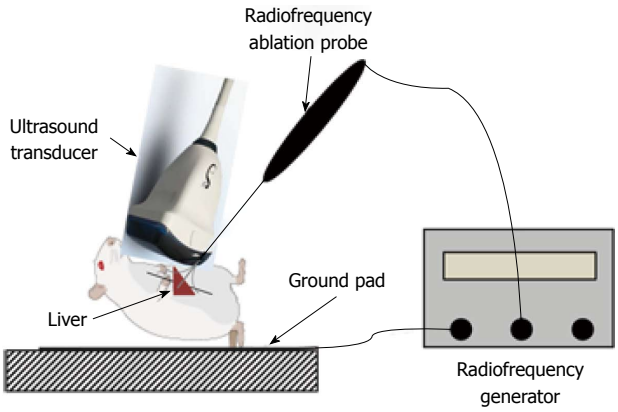

Figure 1 Experimental setup employed in this study. Radiofrequency ablation of the liver was performed in 10 rats under laparotomy.

MATERIALS AND METHODS

Animals

The Animal Care and Use Committee of Tokyo Medical University (TMU) approved the use of animals in this study. Ten male Wistar rats weighing 300-400 g at the time of purchase were studied. All rats received appropriate care from properly trained professional staff in compliance with both the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals as approved by the Animal Care and Use Committee of TMU.

Before RF ablation, the rats were anesthetized by the intraperitoneal injection of sodium pentobarbital (10 mg/kg body weight). The liver was accessed *via* open laparotomy with packing to ensure adequate hepatic exposure. The experimental setup is shown in Figure 1.

RF ablation system

An electrosurgical device (RITA Medical Inc., Mountain View, CA, United States) was used for the RF ablation procedures. The RF ablation electrode of this device consists of a 15-gauge shaft through which multiple sharp tines, each with a diameter of 0.053 cm (0.021 inches, 25 gauge), can be deployed. Fully extended, the tines assume an "umbrella" configuration with the tines spaced at 45º intervals. The electrode was inserted into the liver to a depth of 5 mm and the tines were then deployed, taking care to ensure that the tines remained within the liver parenchyma. RF ablation of the target tissue was performed for 3 min, with the tissue heated to a set temperature of 90 ℃ using a maximum power level of 10 W (Figure 1).

US examinations

US examinations were performed using an Aixplorer US system equipped with a 4-15-MHz linear-array transducer (SuperSonic Imagine, Aix-en-Provence, France) by one hepatologist with 10 years of experience in abdominal US. Fifteen minutes after RF ablation, 3D B-mode US and SWE were performed with a 5-16-MHz dedicated volume transducer. Volume imaging was automatically

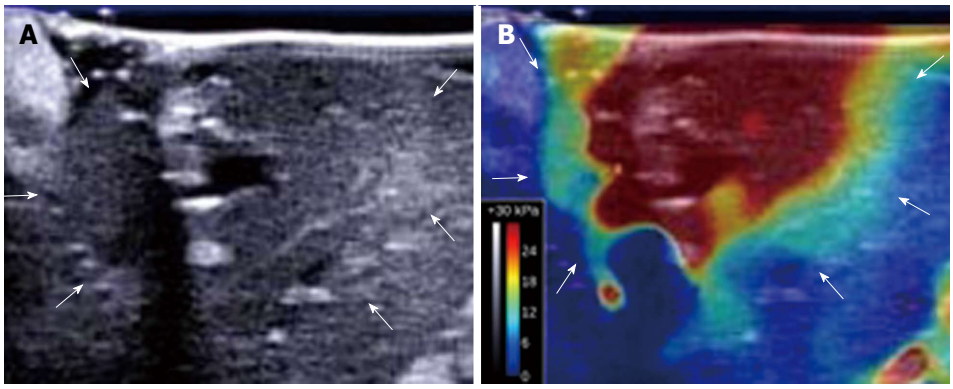

Figure 2 B-mode ultrasound image and shear-wave elastography image of a thermal lesion. A: B-mode ultrasound image of the liver obtained 15 min after ablation shows a poorly defined hypoechoic lesion with a hyperechoic rim (arrows); B: Corresponding shear-wave elastography image of the liver clearly delineates the ablated area (arrows).

performed by slow-tilt movement of the mechanical sector transducer with a sweep angle of 30°. After the US examination was completed, the acquired 3D volume data were rendered and saved in the US system console as a multislice display (interslice distance: 1.10 mm) for off-line analysis, including ablation volume measurement.

Tissue collection and histological analysis

The animals were sacrificed after completion of the US examinations, and the livers were harvested. To permit radiologic-pathologic correlation, US was used to guide cutting of the livers in the proper sectional planes along the course of the ablation electrode. After formalin fixation, the livers were cut by total segmentation and digitized photographs of the cut surfaces were obtained. The photographs were used to estimate the volume of the 10 thermal lesions. For histopathological examination, the liver specimens were embedded in paraffin and cut with a microtome to obtain 3-μm sections. The sections were then stained with hematoxylin and eosin (H-E) or an anti-heat shock protein 70 (Hsp70) antibody (sc-24; Santa Cruz Biotechnology, Dallas, TX, United States) according to the manufacturer's instructions.

SWE and pathological volume measurements

The estimated volumes of the 10 thermal lesions were used to assess the correlation between SWE findings and the digitized photographs of the gross pathological specimens. The estimated volume of each lesion in the gross pathological specimens was calculated by summing the values obtained by multiplying the area of the thermal lesion in each section by the section thickness (3 mm). For SWE, the volume of each lesion was automatically calculated by summing the values obtained by multiplying the area of the thermal lesion in each 2D SWE image by the scan plane interval (1.10 mm). All RF ablation areas in the SWE images as well as in the digitized photographs of the gross pathological sections were manually delineated based on the consensus of two observers.

Statistical analysis

All data are presented as mean ± SD. The differences in the detectability of RF ablation lesion boundaries between SWE and B-mode US imaging were evaluated using the McNemar test. A value of $P < 0.05$ was considered to indicate a statistically significant difference between the groups. The accuracy of the various volume measurements was compared with the reference standard (pathological findings) using multiple linear regression analysis. All statistical analyses were performed with the SPSS 11.0 computer software package (SPSS, Tokyo, Japan).

RESULTS

US findings after RF ablation

For each SWE image, the tissue stiffness of each pixel was displayed as a semitransparent color overlay with a range from dark blue, indicating the lowest stiffness (just over 0 kPa), to red, indicating the highest stiffness (set at 30 kPa). SWE images after RF ablation clearly delineated the ablated area, which is seen as a red area surrounded by a yellow-green area (Figure 2B). The surrounding yellow-green area, which exhibited sinusoidal dilatation accompanied by congestion in the H-E sections, was observed in all RF-ablated lesions. On the other hand, B-mode US after RF ablation showed a poorly defined hypoechoic lesion with a hyperechoic rim (Figure 2A).

All RF ablation areas in the maximal plane of the SWE images as well as in the B-mode images were manually delineated based on the consensus of two observers. Table 1 shows a comparison of the detectability of RF ablation lesion boundaries between SWE and B-mode images. The boundaries of all 10 thermal lesions (100%) were completely delineated by SWE, while only 3 lesions (30%) could be completely delineated in the B-mode images. The other 7 lesions (70%) could only be partially delineated in the B-mode images. The difference in lesion detectability between the two techniques was found to be statistically significant ($P = 0.001$).

In each shear-wave image of the RF ablation area, circular regions of interest (ROIs) measuring 3 mm in diameter were specified in the central red zone, the surrounding yellow-green zone, and the non-ablated zone (3 ROIs in each zone). The mean tissue stiffness values of

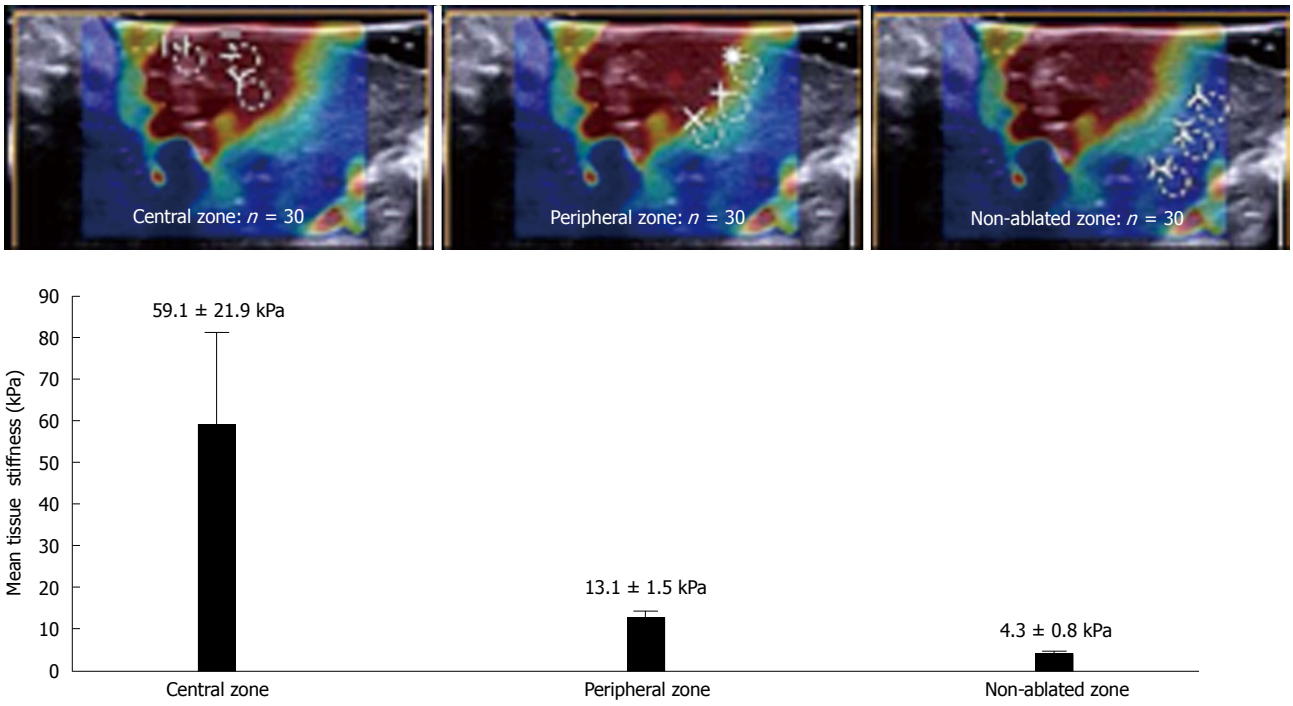

Figure 3 Liver tissue stiffness values of the thermal lesions. Mean tissue stiffness of the central zone, peripheral zone, and non-ablated zone of the livers after radiofrequency ablation. The images at the top show examples of how the regions of interest in each zone were selected.

Table 1 Boundary detection of thermal lesions using B-mode ultrasound and shear wave elastography *n* (%)

Boundary detection	B-mode (n = 10)	SWE (n = 10)
Complete	3 (30)	10 (100)
Partial	7 (70)	0 (0)

All radiofrequency (RF) ablation lesions visualized by shear-wave elastography (SWE) and B-mode ultrasound were manually delineated based on the consensus of two observers. Complete: Complete delineation of RF ablation lesion boundaries; Partial: Partial delineation of RF ablation lesion boundaries.

the central zone, the peripheral zone, and the non-ablated zone were 59.1 ± 21.9 kPa, 13.1 ± 1.5 kPa, and 4.3 ± 0.8 kPa, respectively (Figure 3).

Pathological findings after RF ablation

In the gross pathological specimens, the regions affected by RF ablation could be easily distinguished from unaffected liver tissue based on their colors after formalin fixation. The heat-coagulated lesions appeared light tan to gray and were consistently observed to have a dark brown rim of vascular congestion (Figure 4A). These gross pathological changes correlated well with the histopathological findings. Specifically, the rim of vascular congestion identified by gross pathological examination was histopathologically characterized by sinusoidal dilatation, hemostasis, hemorrhage, and overexpression of Hsp70 in hepatocytes, which accurately discriminated the heat-coagulated lesions from the unaffected normal liver tissue in all samples (n = 10) (Figure 4B, C, and D). A high correlation was observed between the volumes depicted by SWE and those estimated by gross pathological examination (r^2 = 0.9305) (Figure 5).

DISCUSSION

The results of the present study using normal rat livers demonstrate that the depiction of thermal lesions by SWE agrees closely with the findings of gross pathological examination. SWE is therefore expected to be useful for clearly visualizing thermal lesions and obtaining quantitative volume estimates. Comparison between the ablation volumes depicted by SWE and those estimated by gross pathological examination showed a high correlation (r^2 = 0.9305). These findings suggest that 3D SWE may prove to be an accurate and convenient tool for monitoring early treatment effects after RF ablation.

At present, contrast-enhanced US[7], contrast-enhanced computed tomography[8], and magnetic resonance imaging[9] are considered to be suitable methods for depicting ablation zones by demonstrating the absence of contrast enhancement when performed at an appropriate time after the ablation procedure. However, in order to perform virtual real-time monitoring of an ablation procedure, repeated contrast injection is required because there is only a short time frame in which the lesion is detectable before equilibrium is reached. These modalities are therefore unsuitable for real-time monitoring during RF ablation procedures, making it difficult to precisely evaluate the ablation zone at the time of treatment. Moreover, an enhanced rim is usually observed in contrast images, which may be difficult to differentiate from an enhanced rim indicating an area of residual untreated

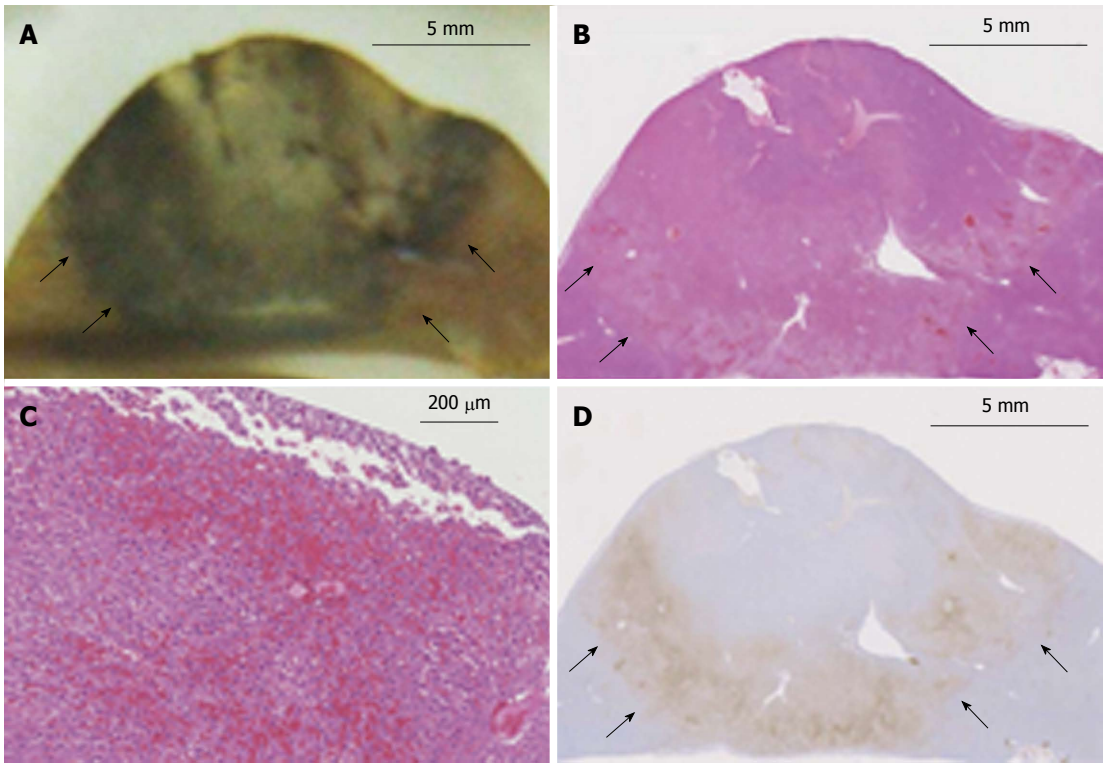

Figure 4 Histopathological findings of a thermal lesion. A: Gross pathological examination of a radiofrequency (RF)-ablated liver shows a core area of white coagulation and a surrounding dark rim of hemorrhagic tissue (arrows). Scale bar: 5 mm; B, C: H-E staining of an RF-ablated liver shows a hemorrhagic rim surrounding the ablated area (arrows), with sinusoidal dilatation and congestion associated with cellular dissociation. Scale bar in (B): 5 mm, and in (C): 200 μm; D: Hsp70 staining of the hemorrhagic rim in (B) shows a clearly demarcated band of Hsp70 expression (arrows), which is suggestive of the presence of thermally damaged cells within the rim. Scale bar: 5 mm.

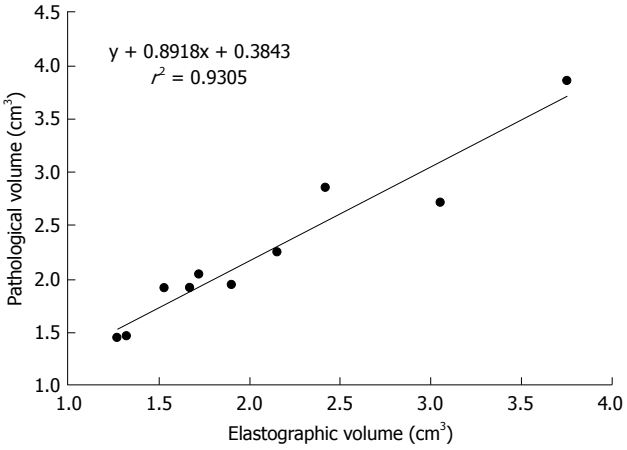

Figure 5 Correlation between shear-wave elastography and gross pathological findings. The correlation between the volume of liver ablation depicted by 3D shear-wave elastography and that estimated by gross pathological examination was 0.9305, which is highly significant.

tumor. For these reasons, SWE may prove to be a more useful method for real-time monitoring in RF ablation procedures.

The H-E staining technique relies on visual examination of the cell membranes and intracellular structures to assess cell viability. Morimoto *et al*[8] have shown that H-E staining provides inconsistent results regarding the extent, or completeness, of tissue necrosis when performed less than 24 h after the application of the necrosis-inducing agent. Therefore, histochemical staining methods, such as those employing lactate dehydrogenase, malate dehydrogenase, and nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase, are thought to be superior to H-E staining for identifying irreversible cellular damage after RF ablation. Accordingly, Morimoto *et al*[8] also evaluated heat-coagulated lesions after RF ablation by analyzing cell viability within the lesions using histochemical staining (lactate dehydrogenase, malate dehydrogenase, and NADPH diaphorase). They found that the regions of heat coagulation (*i.e.*, the central areas of the ablated lesions) were not stained in any of the samples, suggesting 100% cellular destruction within the lesion. Moreover, the surrounding hemorrhagic rim (*i.e.*, the peripheral part of the ablated lesion) was also not histochemically stained in any of the samples, suggesting the disappearance of viable cells from the rim as well. Based on these findings, we also assumed that viable cells were absent from the surrounding narrow rim of vascular congestion observed in our study. Interestingly, a thick rim of Hsp70 staining, which is a marker of thermal damage, was noted in the area peripheral to the ablation zone, which corresponded to the congestive rim. Hsp70 may therefore be a useful biomarker for assessing cell viability after thermal damage[10].

In our study, the mean tissue stiffness of the hemorrhagic rim was 13.1 ± 1.5 kPa, and our findings suggest that this may be an appropriate threshold value for deter-

mining irreversible cellular destruction. This value may also be of clinical significance because an incompletely ablated area should be softer (*i.e.*, less than 13.1 kPa) and show higher contrast than surrounding completely ablated areas, which may provide useful information for guiding a second round of RF ablation to treat the incompletely ablated area.

It should be noted that our study suffers from a number of limitations. First, the study was performed in normal rat livers. Similar studies should be performed in a liver tumor model or in cirrhotic livers to increase the clinical relevance of the findings. Second, cell viability was assessed only by H-E staining and by immunohistochemical analysis of Hsp70. Assessment of cell viability using more reliable methods should be performed in future studies. Third, at present, contrast-enhanced computed tomography and magnetic resonance imaging are considered to be suitable methods for depicting ablation zones by demonstrating the absence of contrast enhancement, but we did not compare SWE against these modalities in our study. Nevertheless, SWE has been shown to be an effective, safe, and cost-efficient tool for evaluating tumor treatment in the interventional suite immediately after ablation.

In conclusion, 3D SWE is a reliable noninvasive technique that may allow the real-time assessment of treatment efficacy immediately after RF ablation procedures. The threshold value for determining remaining cell viability was found to be 13.1 ± 1.5 kPa.

COMMENTS

Background

The ability to assess the completeness of radiofrequency (RF) ablation during or immediately after the procedure would be of great clinical value. If three-dimensional (3D) shear-wave elastography (SWE) is found to be superior for precise assessment of the ablation area, it would be a better method for assessing the completeness of RF ablation because the administration of contrast agent is not required and both 3D assessment and real-time observation are possible.

Research frontiers

To the best of our knowledge, no other investigators have reported on the usefulness of 3D SWE for evaluation of the ablated area after RF ablation procedures.

Innovations and breakthroughs

3D SWE is a reliable noninvasive technique that may allow the real-time assessment of treatment efficacy immediately after RF ablation procedures. It is superior to B-mode ultrasound (US) in delineating ablated areas in the liver.

Applications

By providing a clearer understanding of how closely the boundaries of thermal lesions visualized by 3D SWE correspond to the actual boundaries of tissue ablation determined by histological examination, this study may lead to improved local ablation therapy in patients with hepatic malignancies.

Terminology

Among the various techniques employed in US elastography, SWE is a highly reproducible method that allows measurement of the propagation speed of shear waves within tissues for the local quantification of tissue stiffness in kilopascals or meters per second.

Peer review

The manuscript titled "Radiologic-pathologic correlation of three-dimensional shear-wave elastographic findings after radiofrequency ablation" regards the evaluation of RF ablation with the SWE compared to simple B mode, which is a generally well-written paper focused on an important topic.

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OBSERVATIONAL STUDY

Role of multi-detector computed tomography for biliary complications after liver transplantation

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Abstract

AIM: To investigate the diagnostic performance of multi-detector computed tomography (MDCT) in detecting biliary complications after orthotopic liver transplantation (OLT).

METHODS: Eighty-three consecutive OLT recipients, who presented with clinical or biochemical signs of biliary complications, underwent MDCT examination. Two experienced radiologists assessed MDCT images in consensus to determine biliary complications. Final confirmation was based on percutaneous transhepatic cholangiography or endoscopic retrograde cholangiography in 58 patients, surgery in four patients, liver biopsy in 10, and clinical and sonography follow-up in 11 patients.

RESULTS: Biliary complications were eventually confirmed in 62 of 83 patients (74.7%), including anastomotic biliary strictures in 32, nonanastomotic biliary strictures in 21, biliary stones in nine (5 with biliary strictures), anastomotic bile leak in five, and biloma in six (all with nonanastomotic strictures, and 2 with biligenic hepatic abscess). Twenty-one patients had no detection of biliary complications. The sensitivity, specificity, accuracy, positive predictive value and negative predictive value of MDCT for detecting biliary strictures were 90.6%, 86.7%, 89.2%, 92.3% and 83.9%, respectively. For detecting biliary stones, anastomotic bile leak and biloma, the sensitivity, specificity, accuracy, positive predictive value and negative predictive value of MDCT were all 100%.

CONCLUSION: MDCT is a useful screening tool for detecting biliary complications after OLT.

Key words: Liver; Transplantation; Biliary; Complications; Computed tomography

Core tip: The value of multi-detector computed tomography (MDCT) in detecting biliary complications after orthotopic liver transplantation (OLT) is conflicting. This study, with 83 OLT recipients suspected of biliary complications, suggests that MDCT is a useful screening tool for detecting biliary complications after OLT. MDCT presented a sensitivity of 90.6% and specificity of 86.7% for biliary strictures, and both sensitivity and specificity of 100% for biliary stones, anastomotic bile leak and biloma. So far, this is the largest sample population to investigate the diagnostic accuracy of MDCT for biliary complications after OLT, which will help us make a treatment decision.

Meng XC, Huang WS, Xie PY, Chen XZ, Cai MY, Shan H, Zhu KS. Role of multi-detector computed tomography for biliary complications after liver transplantation. *World J Gastroenterol* 2014; 20(33): 11856-11864 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i33/11856.htm DOI: http://

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INTRODUCTION

Orthotopic liver transplantation (OLT) is a widely accepted treatment for end-stage liver disease and selected cases of hepatocellular carcinoma. Despite significant improvements in surgical techniques, biliary complications after OLT are still associated with high morbidity and mortality, and still constitute one of the main causes of graft dysfunction or loss of recipients[1]. According to available data, the reported rates of biliary complications range from 5.8% to 30% of liver transplants[2-6], and early detection of these complications and adequate management are crucial for graft and patient survival.

However, early detection of biliary complications after OLT is challenging. Clinical manifestations (such as fever, abdominal complaints, and signs of cholangitis) or biochemical findings are often nonspecific[7], which may relate to a wide range of potential complications, including graft rejection, hepatic arterial thrombosis or stenosis[8,9]. Imaging techniques play a key role in differentiating and evaluating biliary complications after OLT[8-11]. Direct cholangiographic procedures, including endoscopic retrograde cholangiography (ERC) and percutaneous transhepatic cholangiography (PTC), are widely accepted as the gold standards for the diagnosis of biliary complications, which could offer potential therapeutic options simultaneously[9]. However, they are both invasive and carry potential risk of complications, such as bleeding, perforation, sepsis, and even death[3]. Magnetic resonance cholangiography (MRC) is a noninvasive imaging technique, which has been considered as an alternative to invasive cholangiography in many clinical settings for its detailed visualization of the bile duct tree without exogenous contrast[8-13]. However, in some cases, MRC cannot be used for routine examination, especially for patients in poor condition who need to carry monitoring equipment or who cannot hold their breath well. Besides these, the scanning techniques are also important factors that can affect the imaging quality of MRC and the diagnostic accuracy of biliary strictures[3]. These limit the application of MRC in the follow-up of OLT recipients.

With fast scanning speed and high imaging quality, multi-detector computed tomography (MDCT) is considered as the main choice for detecting vascular complications after liver transplantation[11,14,15], which reveals the biliary system at the same time. So, there is the potential to detect biliary complications in one-step MDCT examination, which may help with early detection of biliary complications. However, to the best of our knowledge, there are only a few reports focusing on the role of MDCT for biliary complications after liver transplantation[16,17], and the number of patients was small. Therefore, the aim of the present study was to evaluate MDCT for detecting biliary complications following liver transplantation and comparing findings with ERC and PTC.

MATERIALS AND METHODS

Patients

Between October 2003 and October 2012, 83 consecutive OLT recipients, who presented with clinical or biological signs of biliary complications, underwent contrast-enhancement MDCT examination at our institution. All patients presented with abnormal liver function tests and a variety of clinical symptoms such as fever and cholangitis. Among these 83 patients, 64 were men and 19 were women, with an age range of 27-63 years (mean, 46.2 years). This study was approved by our Institutional Review Board. Informed consent was obtained from all patients.

In liver transplantation procedures, bile duct continuity was established in all cases by a primary duct-to-duct anastomosis (choledocho-choledochostomy) without T-tube stent. The interval between OLT and clinical onset of biliary complications ranged from 7 d to 68 mo (mean, 13 mo). Indications for liver transplantation were as follows: hepatitis B liver cirrhosis (n = 54), hepatitis C liver cirrhosis (n = 3), hepatocellular carcinoma (n = 21), sclerosing cholangitis (n = 2), idiopathic cirrhosis (n = 2), and Budd-Chiari syndrome (n = 1).

MDCT techniques

From October 2003 to December 2008, 46 patients underwent CT scans on a GE LightSpeed Qx/I CT scanner (General Electric Medical Systems, Milwaukee, WI, USA) (early-stage MDCT). After December 2008, 37 patients underwent CT on a Toshiba Aquilion ONE CT scanner (Toshiba Medical Systems, Otawara, Tochigi, Japan) (late-stage MDCT). Patients routinely received plain and double-phase contrast-enhanced CT covering the entire craniocaudal extent of the liver and all the vascular anastomoses. After the plain scan, non-ionic iodinated contrast medium (iopromide, Ultravist 300; Schering, Berlin, Germany) was injected *via* an antecubital vein at a dose of 2 mL/kg at a rate of 4 mL/s through a power injector. Contrast-enhanced CT images were obtained during the hepatic arterial phase with a 25-s delay and the portal venous phase with a 65-s delay after initiation of contrast injection.

Technical parameters for the CT examinations on GE LightSpeed Qx/I were as follows: beam collimation 16 mm, high-speed scan mode (pitch, 6), 5.0-mm slice thickness, 2.5-mm reconstruction slice thickness, and 1.0-mm intervals. Technical parameters on the Toshiba Aquilion ONE CT scanner were as follows: beam collimation 16 mm, 0.5-mm slice thickness and intervals, 1.0-mm reconstruction slice thickness, and 0.5-mm intervals. All CT scanning was performed in the supine position at 240 mA and 120 kV with a standard algorithm.

Further data processing was performed on an Advantage workstation 4.0 (General Electric Medical Systems) or a prototype workstation (Toshiba) equipped with software allowing generation of the oblique reformat. One radiologist experienced in the oblique reformat processed

Table 1 Multi-detector computed tomography and final diagnosis in patients with orthotopic liver transplantation with clinical or biochemical evidence of biliary complications

Diagnosis	MDCT (83 patients)	Final (83 patients)
Normal	21	7
Anastomotic strictures	34	32
Nonanastomotic strictures	18	21
Anastomotic bile leakage	5	5
Biliary stones	4	4
Other complications		
Rejection of the transplanted liver	0	6
Recurrent viral hepatitis	0	4
Transplantation-related lymphoproliferation	0	1
Postoperative hilar and portal fluid	0	2
Pancreatitis	1	1

MDCT: Multi-detector computed tomography.

all images to reveal the biliary system. All these images were stored on the hard disk memory of the workstation for subsequent image analysis.

Image interpretation and analysis

The MDCT transverse images and the oblique reformat images were interpreted in conference by two experienced abdominal radiologists who were blinded to patient identification and all the surgical, pathological and cholangiographic findings. All images were retrospectively evaluated to detect the presence of biliary dilatation, biliary strictures, intra- or extrahepatic fluid collection, and biliary stones. Intrahepatic bile ducts were considered dilated if the maximum diameter was > 3 mm[10,13]. Extrahepatic bile ducts (common hepatic and common bile ducts) were considered dilated if measuring > 8 mm[10,13]. For biliary strictures, the site (anastomotic or nonanastomotic) was decided. A round or irregular slightly high or high-density focus inside the bile duct was termed biliary stones. In the case of inter-observer disagreement, a final diagnosis was reached by consensus.

On the basis of MDCT findings, biliary complications were classified as biliary strictures, anastomotic bile leakage, biloma, biliary stones, and other complications. Biliary strictures were further subclassified as anastomotic and nonanastomotic. Anastomotic strictures were defined as a focal stricture at the site of the biliary anastomosis[8,13]. Nonanastomotic strictures were defined as one or multiple strictures of the extrahepatic bile duct, intrahepatic bile ducts (including the hepatic duct confluence), or both[8,13]. Anastomotic bile leakage was defined as a variable amount of pericholedochal fluid collection having direct communication with biliary anastomosis. Biloma was defined as an intrahepatic fluid collection communicating with dilated intrahepatic bile ducts.

Diagnostic confirmation was obtained by direct cholangiography in 58 patients (PTC in 35 and ERC in 23); surgery in four (retransplantation in 2 and hepaticojejunostomy in 2); liver biopsy in 10; and clinical and sonography follow-up of at least 6 mo in 11 patients. The ERC and PTC images were interpreted in conference by two other observers (a radiologist and a surgeon), who used all the clinical, laboratory, endoscopic and PTC imaging data during the review.

Statistical analysis

The MDCT findings were compared with the PTC, ERC, surgical, liver biopsy or imaging follow-up results and defined as true positives when they correctly detected biliary complications confirmed by the final diagnosis reference standards; false positives when they were not confirmed by PTC, ERC, surgery, liver biopsy or imaging follow-up; false negatives when complications were detected by PTC, ERC, surgery, liver biopsy or imaging follow-up were not detected by MDCT; or true negatives when the absence of complications was confirmed by PTC, ERC, surgery, liver biopsy or imaging follow-up. The sensitivity, specificity, accuracy, positive predictive value and negative predictive value of the MDCT findings were determined by the reviewers.

RESULTS

Final diagnostic confirmation

A total of 62 biliary complications were eventually confirmed in the 83 patients (74.7%) by PTC, ERC, surgery, liver biopsy or clinical and sonography follow-up, including anastomotic strictures in 32 patients (38.6%), nonanastomotic strictures in 21 (25.3%), biliary stones in nine (10.8%; 5 with biliary strictures), anastomotic bile leakage in five (6.0%), and biloma in six (7.2%) (2 of the 6 patients with nonanastomotic strictures developed biligenic hepatic abscess). Twenty-one patients had no detection of OLT-related biliary complications: seven showed no abnormality, and 14 had other clinical problems, which raised clinical suspicion of a biliary condition, including graft rejection (n = 6), recurrent viral hepatitis (n = 4), transplantation-related lymphoproliferation of hilar and portal area (n = 1), fluid collections in hepatic hilar and portal area (n = 2), and pancreatitis (n = 1) (Table 1).

MDCT findings

Biliary strictures: In 32 patients with anastomotic strictures, 30 were correctly detected by MDCT, who presented with a short stricture at the anastomosis, with notable biliary dilation above the stricture (Figure 1). The site of the dilated bile duct was intrahepatic in 30 patients (93.8%) and extrahepatic in 27 (84.4%). Two patients with anastomotic strictures were missed at early-stage MDCT (false-negative) due to the absence of biliary dilation. However, ERC confirmed both of them. False-positive findings also occurred in two patients in early-stage MDCT, who presented with mild intra- and extrahepatic biliary dilation and fluid collection in the hilar and portal area. Among 32 patients with anastomotic strictures, five (15.6%) had biliary stones concomitantly, and three (9.4%)

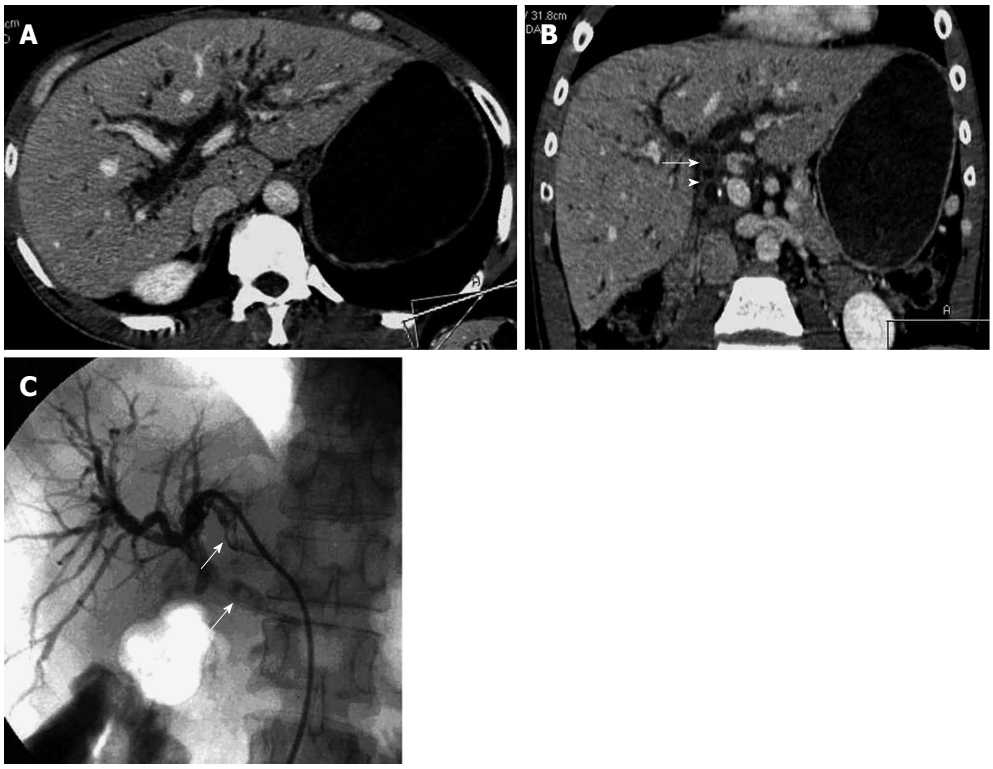

Figure 1 A 36-year-old man with obstructive jaundice due to anastomotic biliary strictures 5 mo after orthotopic liver transplantation. Computed tomography oblique reformat images (A, B) display biliary dilation at the intrahepatic and hepatic hilar bile ducts, biliary stone (arrow) and anastomotic strictures (arrowhead); C: Percutaneous transhepatic cholangiography demonstrates biliary dilation, anastomotic obstruction, and filling defect (arrow) in the common bile duct and the left hepatic duct which caused by biliary stone or sludge.

were complicated with hepatic artery stenosis.

MDCT correctly identified 16 of the 21 cases with nonanastomotic strictures (Figure 2) who presented with one or multiple strictures at the extrahepatic bile duct, the intrahepatic bile ducts (including hepatic confluence), or both. MDCT detected dilation of the intrahepatic bile ducts in 18 patients (85.7%), extrahepatic bile ducts in seven (33.3%), and none in three. Three patients without dilatation were missed (false-negative) by MDCT (one of them occurred in early-stage examination), whose strictures only involved the confluence sites of bile ducts with or without biliary sludge in ERC. Two patients with extrahepatic nonanastomotic strictures were interpreted as anastomotic strictures in early-stage MDCT. There were two false-positive results in two patients with normal bile ducts, who presented with slight intrahepatic biliary dilation in early-stage MDCT. Of the 21 patients with nonanastomotic strictures, 6 (28.6%) were complicated with biloma, and 12 (57.1%) had hepatic artery stenosis.

Biliary stones: MDCT correctly detected all of the cases with biliary stones (n = 9). The stones were typically seen as a round or irregular slightly high or high-density focus inside the dilated bile ducts (Figure 3), and were identified alone (n = 4) or associated with anastomotic strictures (n = 5).

Anastomotic bile leakage: In five patients with anastomotic bile leakage, the observers accurately described the presence of fluid collection adjacent to the anastomotic region (Figure 4), but could not demonstrate active leakage. Anastomotic bile leakage was eventually confirmed by PTC in all five patients.

Biloma and biligenic hepatic abscess: Biloma was found in six patients. It was an intrahepatic bile collection (bile lakes), which appeared as a well-circumscribed rounded lesion with fluid-density of variable size on MDCT. MDCT revealed the communication between the biloma and the dilated bile ducts. Among the six patients with biloma, two developed biligenic hepatic abscess due to biliary infection (Figure 5). All six patients with biloma had nonanastomotic biliary strictures and concomitant hepatic arterial stenosis.

Global results

Of the 53 patients with biliary strictures, 48 were correctly identified by MDCT (sensitivity, 90.6%) and 5 were missed (false-negative). MDCT correctly identified the absence of biliary strictures in 26/30 patients (specificity, 86.7%) and 4 were misdiagnosed (false-positive). The accuracy, positive predictive value and negative predictive value of MDCT in detecting biliary strictures were 89.2%, 92.3% and 83.9%, respectively. In differentiating anastomotic from nonanastomotic strictures, 46 of 53 patients (86.8%) with biliary strictures were correctly identified by MDCT. Missed diagnosis occurred in 5 patients. The other 2 patients with nonanastomotic stric-

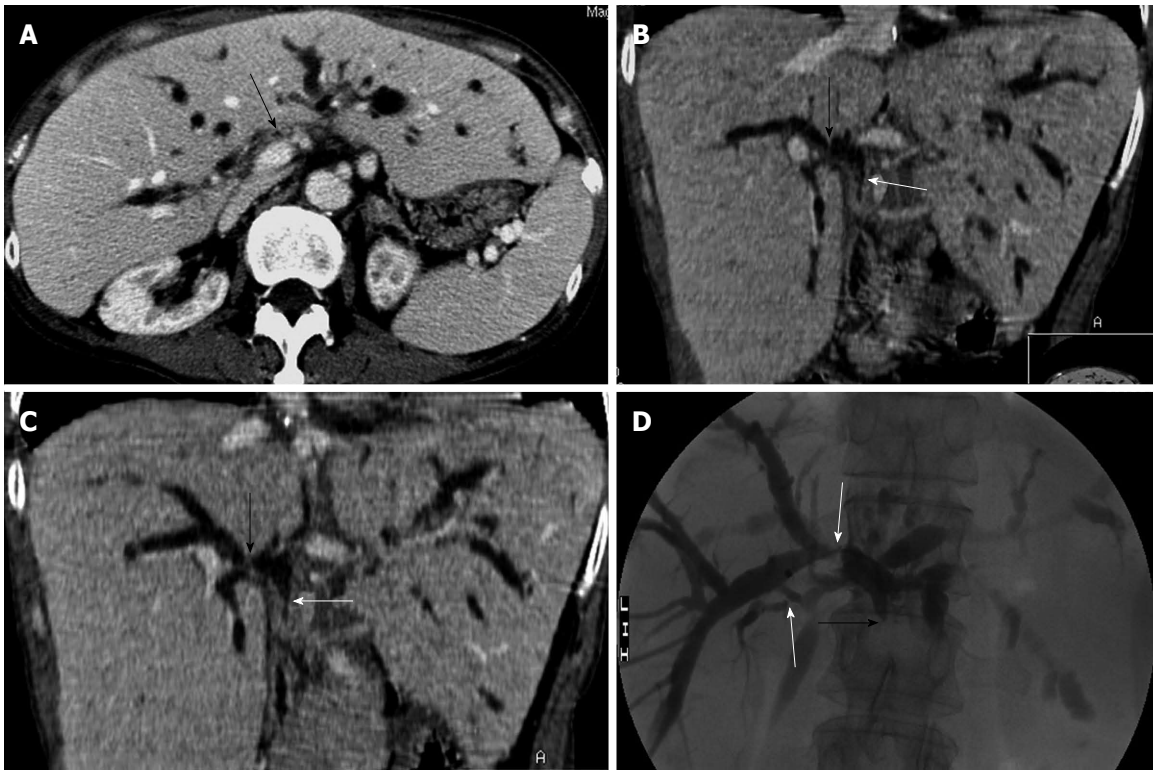

Figure 2 A 66-year-old women with obstructive jaundice due to nonanastomotic biliary strictures 11 mo after orthotopic liver transplantation. A: Transverse computed tomography (CT) image on the portal venous phase shows marked stenosis of the hepatic hilar bile duct (arrow) and dilation of the intrahepatic bile ducts; B,C: CT oblique reformat image shows biliary stenosis at the hepatic bifurcation (black arrow) and the donor common duct (white arrow); D: Percutaneous transhepatic cholangiography demonstrates the stenosis of intrahepatic bile duct and hepatic bifurcation (white arrow), and complete occlusion of the donor common duct (black arrow).

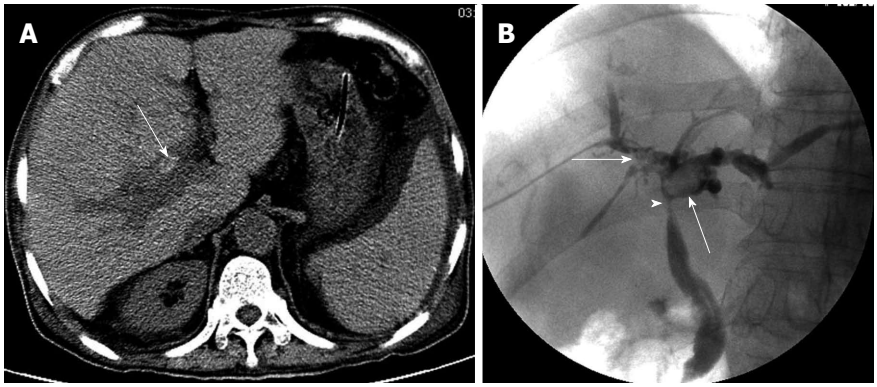

Figure 3 A 57-year-old man with jaundice due to biliary stones and anastomotic biliary strictures 13 mo after orthotopic liver transplantation. A: Transverse plain computed tomography image shows a high-density focus at the hepatic hilar region (arrow); B: Percutaneous transhepatic cholangiography demonstrates filling defect (arrow) caused by biliary stone or sludge in the hepatic hilar bile ducts, and anastomotic biliary strictures (arrowhead).

tures were misdiagnosed as anastomotic strictures by MDCT. In evaluating other biliary complications, including biliary stones, anastomotic bile leak and biloma, the sensitivity, specificity, accuracy, positive predictive value and negative predictive value of MDCT were all 100%. The value of MDCT in detecting biliary complications in OLT patients is shown in Table 2.

DISCUSSION

Biliary strictures are the most common type of late biliary complications after transplantation, which account for 85.5% (53/62) of biliary complications in our study. Elevation of liver enzymes and hyperbilirubinemia in OLT recipients may be caused by a variety of reasons, such as graft rejection, recurrence of underlying liver disease, biliary strictures and/or biliary stones. Imaging evaluation is always necessary to rule out biliary strictures in these patients. Usually, biliary strictures occur from 3 to 5 mo after transplantation, so direct cholangiography *via* post-surgical T-tube is always impossible. Although sonography is a simple method for noninvasive screening test,

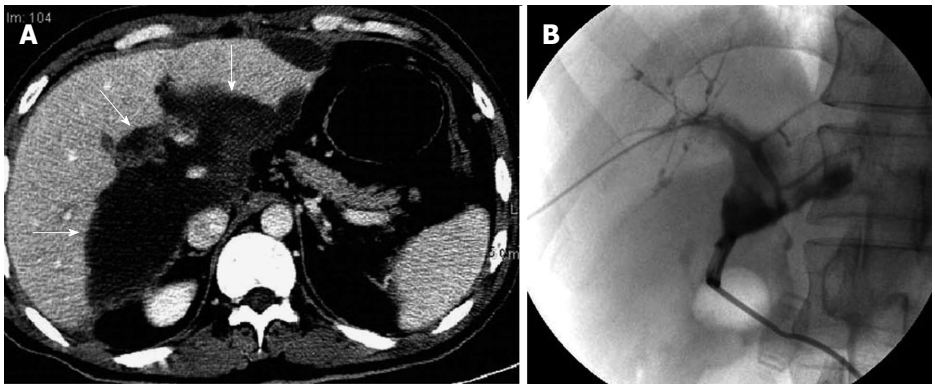

Figure 4 A 32-year-old man with fever due to anastomotic bile leakage 12 d after orthotopic liver transplantation. A: Transverse computed tomography image in the portal venous phase shows a bulk of fluid collection at the hepatic hilar region (arrow); B: Percutaneous transhepatic cholangiography demonstrates anastomotic bile leakage.

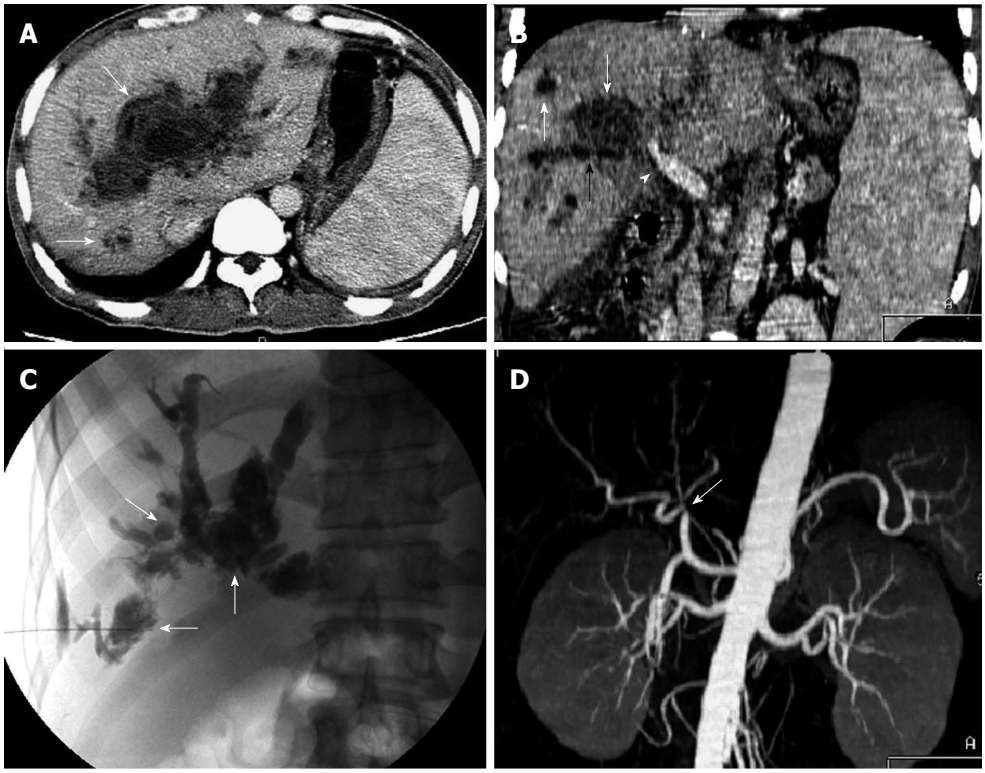

Figure 5 A 46-year-old man with jaundice and fever due to biloma or biligenic hepatic abscess 4 mo after orthotopic liver transplantation. Transverse computed tomography (CT) image in the portal venous phase (A) and oblique reformat image (B) show multiple biloma or biligenic hepatic abscess (white arrow) communicating with the dilated bile ducts (black arrow), and stenosis of the hepatic hilar bile duct (arrowhead); C: Percutaneous transhepatic cholangiography demonstrates biliary dilation, necrosis, and abscess cavity (contrast medium pool) (arrow); D: CT angiography shows hepatic arterial anastomotic stenosis (arrow).

its low sensitivity and high rate of false-negative results in detecting biliary strictures[18-20] limit its usefulness in evaluating biliary complications for OLT recipients. Sensitivity and specificity of MRC for post-transplant biliary complications are reported to be high, however, its diagnostic performance in detecting biliary strictures is often affected by the technique and patients' conditions[8-13]. It has been reported that MDCT can demonstrate biliary complications after liver transplantation, however, the detailed results are not available[11,14]. To the best of our knowledge, only one study reported the diagnostic value of CT on biliary strictures after liver transplantation, however, the small study population could not sufficiently illuminate the potential role of CT[16]. Therefore, the performance of MDCT in detecting biliary strictures needs to be further established.

In our study, MDCT presented a sensitivity of 90.6%, specificity of 86.7%, accuracy of 89.2%, positive predictive value of 92.3%, and negative predictive value of 83.9% for the detection of biliary strictures in 83 post-OLT recipients who received MDCT examination. Compared with Zoepf *et al*[16], our study revealed a higher

Table 2 Value of multi-detector computed tomography in detecting biliary complications for orthotopic liver transplantation recipients

Biliary complications	True-positive (*n*)	True-negative (*n*)	False-positive (*n*)	False-negative (*n*)	Sensitivity	Specificity	Accuracy	Positive predictive value	Negative predictive value
Biliary strictures	48	26	4	5	90.6%	86.7%	89.2%	92.3%	83.9%
Biliary stones	9	74	0	0	100%	100%	100%	100%	100%
Anastomotic bile leakage	5	78	0	0	100%	100%	100%	100%	100%
Biloma	6	77	0	0	100%	100%	100%	100%	100%

The reference standards used for the final diagnosis are given in the text.

sensitivity and specificity. This may be attributed to the improvements of MDCT techniques, which allow the thinner scans and post-processing of bile ducts in any desired planar with oblique reformat. The combination of the transverse MDCT images and oblique reformat images can help to visualize the bile duct tree and the sites of biliary strictures.

Suprastenotic dilation of the bile ducts is an important sign for the detection of biliary strictures on MDCT. In this study, among the 53 patients with biliary strictures, 48 (90.6%) presented with suprastenotic biliary dilatation with a variable degree on MDCT. However, the absence of dilatation on MDCT could not completely deny the existence of biliary strictures. All five patients who were missed (false-negative) by MDCT in this study did not present with biliary dilation. This may have been due to the fact that the marked thickened bile duct wall limited its dilation, or that a denervated donor liver that developed reperfusion or ischemic injury during preservation may show no physiological response to the intraductal pressure[21-23]. Some recipients with biliary strictures do not develop bile duct dilation even in high-grade stenosis after OLT[23]. Therefore, if biliary strictures are highly suspected and MDCT does not provide any evidence of biliary dilation, direct cholangiography (ERC or PTC) should be performed to rule out biliary strictures. It is worth noting that, in some patients, slight biliary dilation is not associated with biliary obstruction. In our study, four patients with slight biliary dilation on MDCT were misdiagnosed as biliary strictures. Fluid collection in the hilar area may be attributed to two of them, which affected the visualization of biliary anastomosis. The cause of biliary dilatation in the other two cases was unclear. Some researchers presumed that this might result from papillary dyskinesia due to devascularization or denervation of the papilla of Vater during transplantation[24].

Biliary strictures are classified as anastomotic or nonanastomotic strictures according to their site. The identification of the type of biliary strictures can help us determine the therapeutic schedule. For the patients only with anastomotic strictures, balloon dilation or stent placement through endoscopic or percutaneous transhepatic methods often can bring good therapeutic results[25,26]. However, for most of the patients with nonanastomotic strictures, endoscopic and/or percutaneous transhepatic biliary drainage or balloon dilation may not reverse the bile duct injuries, and retransplantation may be eventually required for a large portion of them[27,28]. In our study, MDCT could correctly identify the type of strictures in 46 of 53 patients (86.8%), including 30 patients with anastomotic strictures and 16 with nonanastomotic strictures, which provided useful information for the treatment.

Our study further demonstrated that hepatic artery stenosis was an important cause of biliary strictures. In our series, MDCT found hepatic artery stenosis in 57.1% of patients with nonanastomotic strictures, and in 15.6% of patients with anastomotic strictures. MDCT can accurately detect hepatic artery complications, with a sensitivity of 100%, specificity of 89%, and accuracy of 93%[29]. Early diagnosis of hepatic artery stenosis by MDCT may allow successful treatment, with urgent surgical revascularization of the graft or with percutaneous angioplasty, and avoid retransplantation before the development of severe hepatic failure[30-32].

Biliary stones are another important complication after OLT, which usually occur with biliary strictures. MDCT was effective in detecting biliary stones, whose sensitivity, specificity and accuracy were all 100% in our study.

Most anastomotic bile leakages occur in the early period after OLT, and > 70% occur within the first month. In our study, all the anastomotic bile leakage appeared as pericholedochal circumscribed fluid collections on MDCT. It was difficult to distinguish a circumscribed perianastomotic ascitic or postoperative fluid from bile leakage. However, if a fluid collection adjacent to the anastomotic region is detected, anastomotic bile leakage should be considered and ERC or PTC performed for the straight demonstration of active leakage[8,10]. In our study, five patients with anastomotic bile leakage were eventually confirmed by PTC.

Biloma mainly relates to the necrosis of bile duct wall due to hepatic arterial insufficiency, which usually occurs with biliary strictures. In this study, MDCT correctly revealed all six patients with biloma and their complicated nonanastomotic strictures and hepatic arterial stenosis. Two of them presented with biligenic hepatic abscesses due to secondary infection of biloma, and both received retransplantation after CT.

The major drawback of MDCT is that MDCT cannot

directly display the bile duct tree and the sites of biliary strictures, as MRC, ERC or PTC can. However, this does not affect the ability of MDCT in the diagnosis of biliary strictures, especially for a CT scanner with 0.5-mm quantum detector, which can achieve 0.35 mm × 0.35 mm × 0.35 mm isotropic voxel size for fine-detail imaging. In our study, for all patients who received late-stage MDCT, raw data were acquired with 0.5-mm slice thickness and intervals. Based on those, bile duct reconstruction on any desired planar with oblique reformat could clearly visualize the bile duct tree and the sites of biliary strictures, although they are not as intuitive as those on MRC images. This might be the reason why false-positive and false-negative cases of biliary strictures were fewer in late-stage than early-stage MDCT (7 patients *vs* 2 patients). Similar to MRC, it is still difficult to measure precisely the length and degree of strictures based on MDCT images. In contrast, ERC and PTC demonstrate more advantages in showing the site, length and grade of the biliary strictures. Besides these, the inherent drawbacks of MDCT are the applied radiation dose and intravenous application of iodinated contrast medium, which are harmful to the recipients, especially in those with graft or renal dysfunction after transplantation.

In conclusion, MDCT is a useful imaging procedure in the detection of biliary complications after liver transplantation. Biliary dilation is an important sign for the detection of biliary strictures on MDCT, but the absence of dilation cannot rule out biliary strictures. For those who present with symptoms of biliary obstruction but without evidence of biliary dilation on MDCT, ERC or PTC should be considered as early as possible. Fluid collection of bile leakage, biliary stones and biloma can be clearly detected by MDCT, but it is difficult to reveal active leakage.

COMMENTS

Background

Multi-detector computed tomography (MDCT) is considered as the main choice for detecting vascular complications after orthotopic liver transplantation (OLT), which also demonstrates biliary complications in one-step examination. However, the value of MDCT in the detection of biliary complications after OLT is conflicting.

Research frontiers

Early detection of biliary complications after OLT is challenging. Clinical manifestations or biochemical findings are often nonspecific. This study suggests that MDCT is a useful screening tool for detecting biliary complications after OLT, with high rates of sensitivity and specificity.

Innovations and breakthroughs

This is the largest sample population to investigate the diagnostic accuracy of MDCT for biliary complications after OLT.

Applications

This study suggests the performance of MDCT in detecting biliary complications after OLT, which will provide useful information for guiding the treatment of biliary complications.

Terminology

Biliary complications after OLT usually are classified as biliary strictures, anastomotic bile leakage, biliary stones, biloma, biligenic hepatic abscess, and other complications. The incidence ranges from 5.8% to 30% of liver transplants, and early detection of these complications and adequate management are crucial for graft and patient survival.

Peer review

This was a retrospective study regarding the value of MDCT for the detection of post-OLT biliary complications in 83 OLT recipients. The main value of this study is its large sample population and the point that MDCT can reveal biliary complications with high rates of sensitivity and specificity. The images are also educational even for clinicians not directly involved in liver transplantation.

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OBSERVATIONAL STUDY

Association between serum alpha-fetoprotein levels and fatty liver disease: A cross-sectional study

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Abstract

AIM: To investigate the association between serum alpha-fetoprotein (AFP) levels and fatty liver disease (FLD) in a Chinese population.

METHODS: A cross-sectional study was performed among subjects who presented for a health examination at the First Affiliated Hospital, College of Medicine, Zhejiang University in 2013. FLD was diagnosed based on an ultrasonography examination. Serum AFP levels were measured with a chemiluminescence immunoassay.

RESULTS: Of the 9800 subjects enrolled, 2601 were diagnosed with FLD. Subjects with FLD had higher serum AFP levels than those without the disease. Subjects with high serum AFP levels had a higher prevalence of FLD, metabolic syndrome, and its components. Univariate logistic analysis showed that elevated serum AFP levels were associated with an increased risk of FLD (OR = 1.057, 95%CI: 1.031-1.084). However, after adjusting for covariates, AFP no longer remained significantly associated with the risk factors for FLD.

CONCLUSION: Our results suggest that serum AFP levels are significantly associated with FLD and that AFP acts as a cofactor, but not as an independent factor, for FLD.

Key words: Fatty liver; Alpha-fetoprotein; Association; Obesity; Risk factor

Core tip: Fatty liver disease (FLD) is a common liver disease that may progress to cirrhosis and hepatocellular carcinoma. In this study, we observed that serum alpha-fetoprotein (AFP) levels are significantly increased in subjects with FLD, and that AFP levels are significantly associated with metabolic parameters. Univariate logistic analysis showed that elevated serum AFP levels are associated with an increased risk of FLD. However, multivariate logistic regression analysis showed that AFP is not independently associated with the risk factors for FLD. Our results suggest a significant association between AFP and FLD, as well as suggesting that AFP acts as a cofactor, but not as an independent factor, for FLD.

Xu P, Xu CF, Wan XY, Yu CH, Shen C, Chen P, Xu GY, Li YM.

Association between serum alpha-fetoprotein levels and fatty liver disease: A cross-sectional study. *World J Gastroenterol* 2014; 20(33): 11865-11870 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i33/11865.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i33.11865

INTRODUCTION

Fatty liver disease (FLD) is a common condition that is characterized by significant lipid deposition within the liver parenchyma. FLD could be induced through both alcoholic and non-alcoholic pathways. Non-alcoholic fatty liver disease (NAFLD) is also called metabolic fatty liver disease, and is closely associated with obesity and metabolic syndrome[1]. Alcoholic fatty liver disease, on the other hand, is caused by excessive alcohol consumption[2]. In parallel with the epidemic of obesity, NAFLD has become an epidemic around the world. More than 30% of adults are affected by NAFLD in the United States[3], with a prevalence as high as 20% in developing countries such as China[4]. With increasing consumption of alcohol, alcoholic fatty liver disease has also become a growing public concern worldwide[5].

FLD includes a wide clinical and histological spectrum, including simple steatosis, steatohepatitis, fibrosis, and cirrhosis. Simple steatosis is generally considered to be a benign condition, while steatohepatitis may progress to cirrhosis in up to 20% of patients[6]. FLD may progress to hepatocellular carcinoma (HCC) as well[7,8]. Non-alcoholic steatohepatitis has recently been reported to be the second leading etiology of HCC requiring liver transplantation, and is recognized as the most rapidly growing indication for liver transplantation in patients with HCC in the United States[9].

Alpha-fetoprotein (AFP) is a well-established tumor marker for HCC. The potential association between AFP and FLD has been examined in recent studies. Babali *et al*[10] observed that patients with fatty liver had a significantly increased serum AFP level, with said level being positively correlated with the grade of hepatic steatosis. Kara *et al*[11] compared serum AFP levels in 130 male NAFLD patients with those in 57 healthy male controls, but did not observe any significant association between AFP and histopathological findings in NAFLD patients. Polyzos *et al*[12] did not observe any positive association between AFP and NAFLD. The inconsistency of these studies may arise from differences in study population, sample size, and diagnostic methods for FLD.

In this study, we performed a large-sample, cross-sectional survey to analyze the association between AFP and FLD in a Chinese population.

MATERIALS AND METHODS

Study population

This study was performed among adults who presented for their annual health examinations at the First Affiliated Hospital, College of Medicine, Zhejiang University in 2013. The analyses were limited to participants who had full records of anthropometric and biochemical data, as well as results of hepatic ultrasonography examination. Pregnant women and subjects with a self-reported history of chronic viral hepatitis, cirrhosis, or malignant disease were excluded from the analysis. A total of 9800 participants (5880 men and 3920 women) with a median (interquartile range) age of 46.0 (39.0-53.0) years were included in the final analysis. All participants were informed verbally about the purpose and design of the study. Written informed consent was not required due to the observational nature of the study. The personal information of each participant was anonymized both at collection and prior to analysis. The study was approved by the Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University.

Clinical examinations

Clinical examinations were performed following standard procedures as previously described[13,14]. All participates were instructed to complete an overnight fast. Body weight (kg) and standing height (cm) were recorded in light, indoor clothing without shoes. Waist circumference was measured using a non-stretchable standard tape at the minimum circumference between the iliac crest and the rib cage. Blood pressure was recorded using an automated sphygmomanometer with the subject in a sitting position.

Approximately 10-mL whole blood samples were collected from each subject, with serum samples being separated for further analysis without freezing. Serum biochemical values were measured using a Hitachi 7600 AutoAnalyzer (Hitachi, Tokyo, Japan) using standard methods. Serum AFP levels were determined with a chemiluminescence immunoassay using an Abbott-Architect Immunoanalyzer (Abbott Laboratories, Abbott Park, IL).

Diagnosis of FLD and metabolic syndrome

FLD was diagnosed based on ultrasonography criteria suggested by the Chinese Liver Disease Association[15]. The criteria are described as follows: FLD is considered to be present if the ultrasound examination shows a diffuse enhancement of near-field echo in the hepatic region and gradual attenuation of the far-field echo combined with any of the following: (1) unclear display of intrahepatic lacuna structure; (2) mild-to-moderate hepatomegaly with a round and blunt border; or (3) color Doppler ultrasonography showing a reduction in the blood flow signal in the liver or a blood flow signal that is difficult to display even when the distribution of the blood flow is normal.

Metabolic syndrome was defined according to the revised ATP Ⅲ criteria of metabolic syndrome in an Asian Study[16]. Metabolic syndrome was considered to be present if subjects fulfilled any three of the following five factors: (1) central obesity: waist circumference $\geqslant$ 90 cm for males and $\geqslant$ 80 cm for females, and/or BMI $\geqslant$ 25 kg/m^2 for both genders; (2) hypertriglyceridemia: triglycerides

Table 1 Comparison of clinical characteristics between the subjects with and without fatty liver disease

	Without FLD[1]	With FLD[2]	*t* value	*P* value
Age (yr)	45.0 (39.0–53.0)	48.0 (41.0–55.0)	8.248	< 0.001
Gender (male/female, *n*)	3748/3451	2132/469	712.005[3]	< 0.001[3]
Body mass index (kg/m^2)	22.92 (2.71)	26.72 (2.72)	61.262	< 0.001
Waist circumference (cm)	80.4 (8.9)	92.7 (7.7)	58.903	< 0.001
Systolic blood pressure (mmHg)	123.8 (17.0)	134.9 (16.1)	29.053	< 0.001
Diastolic blood pressure (mmHg)	75.4 (10.9)	83.3 (10.5)	31.739	< 0.001
Alanine aminotransferase (U/L)	17.0 (13.0–24.0)	29.0 (21.0–43.0)	42.194[4]	< 0.001[4]
Aspartate aminotransferase (U/L)	20.0 (17.0–24.0)	23.0 (19.0–29.0)	25.339[4]	< 0.001[4]
Gamma-glutamyltransferase (U/L)	19.0 (13.0–31.0)	39.0 (25.0–64.0)	39.846[4]	< 0.001[4]
Direct bilirubin (μmol/L)	3.0 (2.0–4.0)	3.0 (2.0-4.0)	2.113	0.053
Indirect bilirubin (μmol/L)	9.0 (6.0–11.0)	9.0 (7.0–12.0)	4.706[4]	< 0.001[4]
Triglyceride (mmol/L)	1.10 (0.80–1.58)	1.92 (1.40–2.68)	41.797[4]	< 0.001
Total cholesterol (mmol/L)	4.75 (0.90)	5.08 (0.96)	15.839	< 0.001
HDL cholesterol (mmol/L)	1.19 (0.29)	1.02 (0.23)	26.529	< 0.001
LDL cholesterol (mmol/L)	2.54 (0.62)	2.63 (0.66)	6.19	< 0.001
Fasting blood glucose (mmol/L)	4.72 (4.44–5.02)	5.04 (4.67–5.56)	26.250[4]	< 0.001[4]
Serum uric acid (μmol/L)	317.7 (85.3)	390.8 (83.3)	37.671	< 0.001
Alpha-fetoprotein (μg/L)	2.90 (2.20–3.90)	3.10 (2.40–4.10)	7.653[4]	< 0.001[4]

[1]n = 7199; [2]n = 2601; [3]χ^2 value; [4]Z value. The data are expressed as the mean ± SD or median (interquartile range) depending on the data distribution. FLD: Fatty liver disease; HDL: High-density lipoprotein; LDL: Low-density lipoprotein.

≥ 1.7 mmol/L; (3) reduced high-density lipoprotein cholesterol (HDL-C): HDL-C < 1.03 mmol/L for males and < 1.29 mmol/L for females; (4) elevated blood pressure: systolic blood pressure ≥ 130 mmHg or diastolic blood pressure ≥ 85 mmHg; and (5) elevated fasting blood glucose: fasting blood glucose ≥ 5.6 mmol/L or previously diagnosed type 2 diabetes.

Statistical analysis

Data were expressed as the mean and standard deviation or the median and interquartile range as appropriate. Student's *t*-test, Mann-Whitney *U* test, and χ^2 test were used for comparisons between the groups. Spearman's analysis was used to determine the correlations between serum AFP levels and other metabolic parameters. Univariate and multivariate logistic regression analyses were applied to assess the factors associated with the presence of fatty liver. All of the analyses were performed using SPSS 13.0 software for Windows (SPSS Inc., Chicago, IL). $P < 0.05$ (2-tailed) was considered to be statistically significant.

RESULTS

Clinical characteristics of the subjects

Of the 9800 subjects enrolled in this study, 2601 (26.5%) and 3110 (31.7%) fulfilled the diagnostic criteria of FLD and metabolic syndrome, respectively. The prevalence of metabolic syndrome parameters including central obesity, elevated blood pressure, hypertriglyceridemia, reduced HDL-C, and elevated fasting blood glucose was 45.1%, 44.3%, 31.4%, 52.7%, and 11.3%, respectively.

Clinical characteristics were compared between subjects with and without FLD. As shown in Table 1, subjects with FLD are older and predominantly male. Significantly more unfavorable anthropometric and biochemical variables are observed among subjects with FLD compared with those without FLD. A notable finding is that serum AFP levels are significantly increased in subjects with FLD compared with those without FLD (Table 1), suggesting a potential association between AFP and FLD.

Correlations between AFP and metabolic syndrome

To better understand the association between AFP and FLD, we analyzed the association between AFP and the features of metabolic syndrome, which are closely associated with FLD. Spearman's analysis showed that serum AFP levels are significantly and positively correlated with body mass index, waist circumference, systolic and diastolic blood pressure, triglyceride level, and fasting blood glucose, whereas they are negatively correlated with high density lipoprotein cholesterol (Table 2). These results suggest that subjects with higher serum AFP levels are associated with more unfavorable metabolic parameters.

We also analyzed the association between serum AFP levels and the prevalence of metabolic syndrome and its components. We divided all of the subjects into two groups according to the median level of serum AFP (3.0 μg/L), and observed a significantly higher prevalence of FLD, metabolic syndrome, and its components including central obesity, elevated blood pressure, hypertriglyceridemia, and elevated fasting blood glucose in subjects with serum AFP ≥ 3.0 μg/L compared with those with serum AFP < 3.0 μg/L (Figure 1). These results suggest a significant association between AFP and metabolic syndrome, while also indirectly supporting an association between serum AFP levels and FLD.

Risk factors for the presence of FLD

We further applied both univariate and multivariate logistic regression analyses to assess the risk factors for FLD. In the univariate model, elevated serum AFP levels were observed to be associated with an increased risk of FLD

Table 2 Correlations between alpha-fetoprotein and features of metabolic syndrome

	BMI	WC	SBP	DBP	TG	HDL-C	FBG
r value	0.101	0.17	0.11	0.135	0.17	-0.043	0.086
P value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

BMI: Body mass index; DBP: Diastolic blood pressure; FBG: Fasting blood glucose; HDL-C: High density lipoprotein cholesterol; SBP: Systolic blood pressure; TG: Triglyceride; WC: Waist circumference.

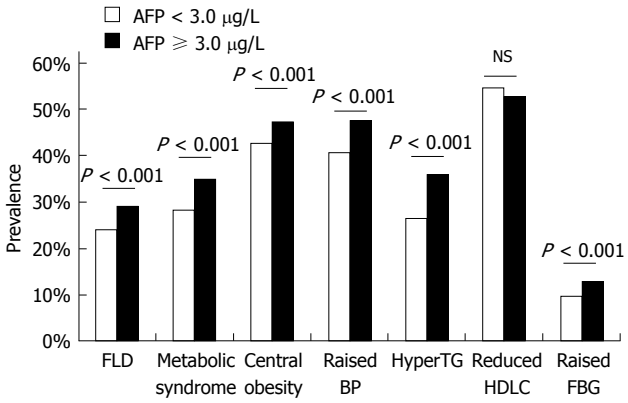

Figure 1 Prevalence of fatty liver disease, metabolic syndrome, and its components in subjects with different serum alpha-fetoprotein levels. Subjects were classified into two groups according to the median level of serum alpha-fetoprotein (AFP) (3.0 μg/L). Significantly higher prevalence of fatty liver disease, metabolic syndrome, and its components including central obesity, elevated blood pressure (BP), hypertriglyceridemia (hyperTG), and elevated fasting blood glucose (FBG) were observed in subjects with serum AFP ⩾ 3.0 μg/L compared with those with serum AFP < 3.0 μg/L. FLD: Fatty liver disease; HDLC: High-density lipoprotein cholesterol; FBG: Fasting blood glucose.

(OR = 1.057, 95%CI: 1.031-1.084; Table 3). However, after adjusting for the 17 variables listed in Table 4 using a multivariate stepwise logistic analysis (Backward: Wald; Entry: 0.05, Removal: 0.10), AFP no longer remained significantly associated with the risk factors for FLD. This result indicates that the relation between AFP and FLD is somehow influenced by other variables.

DISCUSSION

In this study, we aimed to investigate the association between serum AFP and FLD in a Chinese population. Of the 9800 subjects enrolled, the prevalence of FLD was 26.5%. Subjects with FLD exhibited higher serum AFP levels than those without FLD. A significant association between serum AFP levels and metabolic syndrome indirectly supports the link between AFP and FLD. Univariate logistic analysis showed that elevated serum AFP levels are associated with an increased risk of FLD. However, multivariate logistic regression analysis showed that AFP is not independently associated with the risk factors for FLD. These results suggest a significant association between AFP and FLD, as well as suggesting that AFP may act as a cofactor, but not as an independent factor, for FLD.

Table 3 Univariable analysis for factors associated with fatty liver disease

Variables	OR	95%CI	*P* value
Age (yr)	1.017	1.013-1.021	< 0.001
Male gender	4.185	3.748-4.672	< 0.001
Body mass index (kg/m^2)	1.667	1.629-1.706	< 0.001
Waist circumference (cm)	1.187	1.177-1.197	< 0.001
Systolic blood pressure (mmHg)	1.038	1.035-1.041	< 0.001
Diastolic blood pressure (mmHg)	1.068	1.063-1.073	< 0.001
Alanine aminotransferase (U/L)	1.050	1.047-1.053	< 0.001
Aspartate aminotransferase (U/L)	1.039	1.033-1.045	< 0.001
Gamma-glutamyltransferase (U/L)	1.013	1.012-1.015	< 0.001
Direct bilirubin (μmol/L)	1.006	0.982-1.031	0.631
Indirect bilirubin (μmol/L)	1.020	1.010-1.030	< 0.001
Triglyceride (mmol/L)	1.992	1.898-2.091	< 0.001
Total cholesterol (mmol/L)	1.462	1.392-1.534	< 0.001
HDL cholesterol (mmol/L)	0.079	0.065-0.097	< 0.001
LDL cholesterol (mmol/L)	1.248	1.163-1.340	< 0.001
Fasting blood glucose (mmol/L)	1.828	1.724-1.938	< 0.001
Serum uric acid (μmol/L)	1.010	1.009-1.010	< 0.001
Alpha-fetoprotein (μg/L)	1.057	1.031-1.084	< 0.001

HDL: High-density lipoprotein; LDL: Low-density lipoprotein.

Table 4 Multivariable analysis for factors associated with fatty liver disease

Variables	OR	95%CI	*P* value
Age (yr)	1.011	1.004-1.018	0.003
Male gender	1.408	1.160-1.709	0.001
Body mass index (kg/m^2)	1.268	1.218-1.321	< 0.001
Waist circumference (cm)	1.064	1.049-1.080	< 0.001
Diastolic blood pressure (mmHg)	1.020	1.014-1.027	< 0.001
Alanine aminotransferase (U/L)	1.043	1.036-1.051	< 0.001
Aspartate aminotransferase (U/L)	0.953	0.940-0.966	< 0.001
Triglyceride (mmol/L)	1.293	1.226-1.362	< 0.001
HDL cholesterol (mmol/L)	0.411	0.307-0.550	< 0.001
LDL cholesterol (mmol/L)	1.318	1.189-1.461	< 0.001
Fasting blood glucose (mmol/L)	1.338	1.251-1.430	< 0.001
Serum uric acid (μmol/L)	1.004	1.003-1.005	< 0.001

HDL: High-density lipoprotein; LDL: Low-density lipoprotein.

FLD is a common liver disease that affects approximately 27% of the urban adult population in China[17] and an even higher proportion of adults in developed countries[18]. FLD may progress to end-stage liver diseases such as cirrhosis and HCC. A recent study reported that FLD, both alcoholic and non-alcoholic, accounted for 9.2% of all HCC cases diagnosed in Japan between 2006 and 2009[19]. Another recent study also reported that non-alcoholic steatohepatitis was ranked as the second most common etiology of HCC requiring liver transplantation in the United States[9], and non-alcoholic steatohepatitis is the most rapidly growing indication for liver transplantation in patients with HCC in the United States[9]. Postoperative morbidity and 30-d mortality rates were significantly higher in the FLD-related HCC patients than viral hepatitis-associated HCC patients[20]. Based on these findings, screening for HCC in FLD patients would be beneficial, and this may be particularly applicable for

steatohepatitis patients.

AFP is a major serum protein produced by the liver of a fetus. Serum AFP levels peak around the 12th week of gestation and then decline to negligible levels during the first year of life. AFP is observed to be elevated in several non-neoplastic hepatic disorders[21]. A statistically significant increase in serum AFP levels was also observed in patients with fatty liver compared with healthy controls (4.09 ± 1.68 ng/mL *vs* 2.95 ± 0.41 ng/mL, $P = 0.008$) by Babali *et al*[10], and their correlation analysis showed that serum AFP levels positively correlate with the grade of hepatic steatosis[10]. However, there have been other studies which not observe any difference in serum AFP levels between subjects with or without fatty liver[11,12]. The inconsistency among these studies may be explained by the differences in the study population, sample size, and diagnostic methods for FLD.

By analyzing 9800 subjects who presented for their health examination at a large medical center, we observed that subjects with ultrasonography diagnosed FLD have higher serum AFP levels than those without FLD. We also observed that serum AFP levels are significantly and positively correlated with metabolic syndrome and its components. Our univariate logistic regression analyses showed that AFP is significantly associated with the risk factors for FLD. However, AFP no longer remains significantly associated with the risk factors for FLD in the multivariate model. Our results suggest a significant association between serum AFP levels and FLD.

It is interesting to consider why serum AFP levels are elevated in subjects with FLD. Hepatocyte necrosis and subsequent hepatic regeneration is hypothesized to be responsible for the increases in serum AFP levels[21]. Hepatocyte proliferation during liver regeneration is also observed to be associated with dedifferentiation of mature hepatocytes and temporarily increased expression of AFP in the liver[22]. Serum AFP levels may also increase as a result of altered hepatocyte-hepatocyte interaction and the loss of normal architectural arrangements[23]. Although the precise mechanisms remain unclear, our results still have significant clinical implications. Based on the fact that FLD may progress to HCC and that serum AFP levels are elevated in subjects with FLD, monitoring serum AFP levels to screen for HCC in FLD patients has significant clinical importance. Indeed, a study reported that AFP combined with prothrombin induced by a lack of vitamin K or a vitamin K antagonist-Ⅱ (PIVKA-Ⅱ) may be considerably valuable for surveillance of HCC in FLD[24].

Several limitations are acknowledged in this study. First, FLD was diagnosed based on hepatic ultrasonography, which is not sensitive for mild hepatic steatosis; neither is it sensitive for diagnosing steatohepatitis or fibrosis. Therefore, the association of serum AFP levels with histopathological findings in FLD could not be analyzed in this study. Liver biopsy is the gold standard for the diagnosis of FLD, but such a procedure is invasive and may cause complications[25]. Ultrasonography is widely used in large sample studies of FLD because it is widely available, safe, and has acceptable diagnostic value for detecting hepatic steatosis[26]. Second, FLD can be caused through both alcoholic and non-alcoholic etiologies. In this study, we did not record alcohol consumption information in all subjects. Whether AFP is differentially associated with alcoholic and non-alcoholic fatty liver disease remains to be determined. Third, serum insulin levels have not been analyzed in this study. It remains unclear whether serum AFP levels are associated with insulin resistance. Nevertheless, our correlation analysis showed that serum insulin levels were significantly associated with metabolic variables, and subjects with higher serum AFP levels had a higher prevalence of metabolic syndrome and its components. Our results indirectly suggested a potential positive association between serum AFP levels and insulin resistance. Moreover, the degree of apoptosis in hepatocytes is known to be elevated in steatotic livers[27,28]. Thus, it would be valuable to correlate AFP levels with markers of hepatocyte cell death, such as cytokeratin 18 fragments. Further studies are needed to clarify these issues.

In conclusion, our large cross-sectional study shows that serum AFP levels are significantly association with FLD and may act as a cofactor, but not as an independent factor, for FLD. Our results indirectly suggest that it may be worthwhile to monitor serum AFP levels to screen for HCC in FLD patients.

COMMENTS

Background

Fatty liver disease (FLD) is a common chronic liver disease that may progress to cirrhosis and hepatocellular carcinoma. The association between serum alpha-fetoprotein (AFP) levels and FLD remains controversial in the literature.

Research frontiers

The factors associated with the development and progression of FLD have been extensively investigated during recent years.

Innovations and breakthroughs

The authors observed that serum AFP levels are significantly increased in subjects with FLD, and AFP levels are significantly associated with metabolic parameters. Univariate logistic analysis showed that elevated serum AFP levels are associated with an increased risk of FLD. However, multivariate logistic regression analysis showed that AFP is not independently associated with the risk factors for FLD.

Applications

This results show that serum AFP levels are significantly associated with FLD and may act as a cofactor, but not as an independent factor, for FLD. This results indirectly suggest that it may be worthwhile to monitor serum AFP levels for screening of hepatocellular carcinoma in FLD patients.

Terminology

AFP is a major serum protein produced by the liver of a fetus. Serum AFP level may be elevated in patients with hepatocellular carcinoma and several non-neoplastic hepatic disorders.

Peer review

This large cross-sectional study was well-designed, and the methods are valid. The results suggested that serum AFP levels are significantly associated with FLD and that AFP acts as a cofactor, but not as an independent factor, for FLD. Although there were several limitations in this study, as the authors have mentioned in the discussion, the observations are useful and, based on the findings, it is recommended that physicians monitor serum AFP levels to screen for hepatocellular carcinoma in FLD patients.

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OBSERVATIONAL STUDY

Low immediate postoperative platelet count is associated with hepatic insufficiency after hepatectomy

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Abstract

AIM: To investigate the relationship between low immediate postoperative platelet count and perioperative outcome after liver resection in patients with hepatocellular carcinoma (HCC).

METHODS: In a cohort of 565 consecutive hepatitis B-related HCC patients who underwent major liver resection, the characteristics and clinical outcomes after liver resection were compared between patients with immediate postoperative platelet count < 100 × 10^9/L and patients with platelet count ⩾ 100 × 10^9/L. Risk factors for postoperative hepatic insufficiency were evaluated by multivariate analysis.

RESULTS: Patients with a low immediate postoperative platelet count (< 100 × 10^9/L) had more grade Ⅲ-Ⅴ complications (20.5% *vs* 12.4%, $P = 0.016$), and higher rates of postoperative liver failure (6.8% *vs* 2.6%, $P = 0.02$), hepatic insufficiency (31.5% *vs* 21.2%, $P < 0.001$) and mortality (6.8% *vs* 0.5%, $P < 0.001$), compared to patients with a platelet count ⩾ 100 × 10^9/L. The alanine aminotransferase levels on postoperative days 3 and 5, and bilirubin on postoperative days 1, 3 and 5 were higher in patients with immediate postoperative low platelet count. Multivariate analysis revealed that immediate postoperative low platelet count, rather than preoperative low platelet count, was a significant independent risk factor for hepatic insufficiency.

CONCLUSION: A low immediate postoperative platelet count is an independent risk factor for hepatic insufficiency. Platelets can mediate liver regeneration in the cirrhotic liver.

Key words: Thrombocytopenia; Hepatic insufficiency; Hepatocellular carcinoma; Hepatectomy; Hepatitis B

Core tip: Recent animal experiments suggested that platelets not only have a role in hemostasis and thrombogenesis, but can also improve liver function by mediating liver regeneration. Our study found that patients with a low immediate postoperative platelet count < 100 × 10^9/L had more grade Ⅲ-Ⅴ complications, and higher rates of postoperative liver failure, hepatic insufficiency and mortality. In addition, these patients had worse liver function after liver resection, with higher alanine aminotransferase and bilirubin and lower albumin levels. This indicated that platelets could mediate liver regeneration in cirrhotic liver.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most common global cause of cancer-related deaths[1]. Liver resection is performed as first-line treatment in almost all HCC patients[2]. With the refinement of surgical techniques and perioperative management in liver surgery during the last few decades, outcomes after liver resection have improved substantially in recent years, with the operative mortality less than 5% in high-volume centers[3]. However, liver failure or hepatic insufficiency, which usually results in severe outcomes, is more common after liver resection, with an incidence ranging from 1.2% to 32%[4,5]. In Asia, about 80% of HCC cases occur in patients with cirrhosis derived from hepatitis B virus (HBV) infection[1]. Concomitant portal hypertension and thrombocytopenia usually increase the risk of postoperative liver failure and hepatic insufficiency[6,7]. Recent studies on animals demonstrated that platelets had a role not only in blood coagulation[8], but also in liver regeneration[9-12], tissue repair[13] and ischemia/reperfusion injury[14]. Several clinical studies[15-18] have also indicated that preoperative thrombocytopenia is a risk factor associated with postoperative complications and mortality. In addition, immediate postoperative low platelet count has recently been proved to be associated with delayed liver function recovery after partial liver resection for colorectal liver metastases, which indicated that platelets play a critical role in liver regeneration after liver resection[19]. However, the role of immediate postoperative low platelet count in underlying liver damage derived from HBV infection has not been investigated. Here, we report a cohort study to determine the relationship between immediate postoperative platelet count and outcome after partial liver resection in patients with HBV-related HCC.

MATERIALS AND METHODS

Study population

Between January 2009 and March 2013, 574 consecutive HBV-related HCC patients who underwent major liver resection were included in this study. All included patients were diagnosed with HCC by histology and with HBV infection (or a history of HBV infection). All patients underwent surgery when Child-Turcotte-Pugh (CTP) class was A. Medical records containing patient demographics, comorbid conditions, laboratory values, intraoperative parameters and postoperative outcomes were obtained from the West China Hospital of Liver Cancer Registry Database. The protocol was approved by the West China Hospital Ethics Committee and written informed consent was obtained from all patients before inclusion. We excluded 9 patients due to synchronous biliary obstruction, splenectomy or platelet transfusion. This resulted in a total of 565 patients included in the study. All patients had major liver resection which was defined as the resection of more than three liver segments. The platelet count was obtained immediately after surgery (usually upon arrival at the intensive care unit or liver department after surgery, and referred to as the immediate postoperative platelet count), and the patients were stratified into the Low Platelet Group (platelet count $< 100 \times 10^9$/L) and High Platelet Group (platelet count $\geqslant 100 \times 10^9$/L). The aim of this study was to evaluate whether immediate postoperative platelet count was associated with hepatic insufficiency and mortality.

Perioperative management

All patients were managed by the same surgical team. The patients underwent a thorough history, physical examination and routine preoperative laboratory measurements. Routine preoperative imaging examination to evaluate the tumor included contrast computed tomography or magnetic resonance imaging of the abdomen. Echocardiography, chest radiography or computed tomography and pulmonary function tests were carried out if necessary. Patients were operated on under general anesthesia and were explored through an extended right subcostal incision, and intraoperative ultrasonography was used routinely. Hemihepatic vascular inflow occlusion[20] or the Pringle maneuver[21] was used according to the surgeon's preference in most patients. Liver parenchymal transection was performed using the Hooking with ligation method[20] or an ultrasonic dissector with coagulator. Based on preoperative and intraoperative condition, patients were transferred to the intensive care unit for treatment if necessary.

Definition of the parameters measured

The 50-50 criteria[22] defined as prothrombin time < 50% and serum bilirubin level > 50 μmol/L on day 5 after liver resection, were used to define liver failure. Based on the 50-50 criteria, hepatic insufficiency[19] was defined as bilirubin > 50 μmol/L or prothrombin time < 50% at any time point between postoperative day 1 and postoperative day 5. Hepatic insufficiency was used as a surrogate marker for poor liver regeneration[19]. The liver volume removed was calculated as follows: segment Ⅰ: 2%, segment Ⅱ: 8%, segment Ⅲ: 8%, segment Ⅳ: 17%, segment Ⅴ: 17.5%, segment Ⅵ: 15%, segment Ⅶ: 15%, segment Ⅷ: 17.5%. Immediate postoperative platelet count indicated the platelet count obtained on the day of surgery. Mortality was defined as death within 30 d after surgery or death before discharge involving a hospital stay of more than 30 d. The Clavien-Dindo complication classification system[23] was used for postoperative complication grading, and grade Ⅲ-Ⅴ complications were defined as severe complications. Extrahepatic procedures included all other operations, except liver resection, such as bowel resection, adrenalectomy, diaphragm resection and adhesion separation due to reoperation.

Statistical analysis

All statistical analyses were performed using SPSS v17 software (SPSS Inc., Chicago, IL, United States), and statistical significance was set at $P < 0.05$. Continuous

Table 1 Patient characteristics in patients with low or high platelet count after major liver resection *n* (%)

Clinical characteristics	Low platelet group (*n* = 146)	High platelet group (*n* = 419)	*P* value
Male	131 (89.7)	353 (84.2)	0.104
Age > 65 yr	23 (15.8)	51 (12.2)	0.269
Preoperative platelet < 100 × 10^9/L	79 (54.1)	28 (6.7)	< 0.001
ASA grade			0.058
Ⅰ-Ⅱ	117 (80.1)	369 (88.1)	
Ⅲ	24 (16.4)	42 (10.0)	
Ⅳ	5 (3.4)	8 (1.9)	
BMI (kg/m^2), mean (SD)	22.93 (2.80)	22.90 (2.83)	0.941
Esophageal varices	22 (15.1)	47 (11.2)	0.221
Hypertension	30 (20.5)	66 (15.8)	0.184
Cardiovascular disease	3 (2.1)	12 (2.9)	0.822
Pulmonary disease	3 (2.1)	9 (2.1)	1.000
Diabetes mellitus	22 (15.1)	26 (76.2)	0.001
HBsAg	124 (84.9)	330 (78.8)	0.106
HBeAg	22 (15.1)	58 (13.8)	0.714
HBV DNA > 2000 U/mL	51 (34.9)	135 (32.2)	0.548
AST (U/L) > ULN	68 (46.6)	194 (46.3)	0.954
ALT (U/L) > ULN	49 (33.6)	133 (31.7)	0.685
Total bilirubin (μmol/L), median (IQR)	14.5 (10.3-18.6)	12.9 (9.9-18.0)	0.145
Albumin (g/L), median (IQR)	40.9 (43.1-37.6)	40.5 (37.6-43.2)	0.538
Hemoglobin (g/L), median (IQR)	141 (129-150)	140 (127-152)	0.913

BMI: Body mass index; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen; SD: Standard deviation; ULN: Upper limit of normal; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ASA: American Society of Anesthesiologists category; IQR: Interquartile range.

variables were reported as mean (SD) or median (range), and were compared using the Student *t* test for continuous variables with parametric distribution, and the Mann-Whitney *U* test or Kruskal-Wallis *H* test for those with nonparametric distribution. Categorical variables were reported as numbers and percentages, and compared using Pearson's χ^2 analysis or Fisher's exact test. To identify risk factors for hepatic insufficiency, only significant factors associated with hepatic insufficiency in the univariate analysis were entered into the forward stepwise logistic regression analysis.

RESULTS

Patient characteristics and operative details in the low platelet group and high platelet group

In our cohort, 146 (25.8%) patients had immediate low postoperative platelet count (platelet count < 100 × 10^9/L) (Table 1), of whom 79 (54.1%) had low preoperative platelet count. A total of 419 patients had postoperative platelet count > 100 × 10^9/L immediately after surgery. All these patients were in CTP class A when surgery was performed. Patients in the Low Platelet Group had a significantly increased rate of preoperative thrombocytopenia ($P < 0.001$) and diabetes mellitus ($P = 0.001$). The two groups had similar operative procedures (Table 2), except that the Low Platelet Group required more packed red blood cell transfusion ($P = 0.001$) because of greater blood loss ($P = 0.036$). There were no significant differences between the groups regarding the other analyzed parameters (Tables 1 and 2).

Table 2 Intraoperative and postoperative parameters in patients with low or high platelet count after major liver resection *n* (%)

Intraoperative parameters	Low platelet group (*n* = 146)	High platelet group (*n* = 419)	*P* value
Liver volume removed			0.140
< 35%	61 (41.8)	215 (51.3)	
35%-65%	38 (26.0)	91 (21.7)	
> 65%	47 (32.2)	113 (27.0)	
Extrahepatic procedures	33 (22.6)	90 (21.5)	0.777
Liver resection with			0.883
Hooking with ligation	35 (24.0)	103 (24.6)	
Ultrasonic dissection	111 (76.0)	316 (75.4)	
Inflow occlusion	60 (41.4)	191 (45.6)	0.347
Anatomic resection	94 (64.4)	293 (69.9)	0.214
Blood loss (mL), mean (SD)	765 (1011)	583 (436)	0.036
PRBCs transfusion	58 (39.7)	105 (25.1)	0.001
Total complication	63 (43.2)	150 (35.8)	0.114
Ⅲ-Ⅴ grade complication	30 (20.5)	52 (12.4)	0.016
Mortality	10 (6.8)	2 (0.5)	< 0.001
Liver failure	10 (6.8)	11 (2.6)	0.020
Hepatic insufficiency	46 (31.5)	89 (21.2)	0.012
ICU stay (d), median (IQR)	0 (0-1)	0 (0-1)	0.129
Hospital stay (d), median (IQR)	14 (12-18)	13 (11-16)	0.011

PRBC: Packed red blood cell; IQR: Interquartile range; SD: Standard deviation; ICU: Intensive care unit.

Postoperative outcomes in the low platelet group and high platelet group

No significant differences were found in total complications between the two groups. However, compared with the High Platelet Group, the Low Platelet Group had more grade Ⅲ-Ⅴ complications (20.5% *vs* 12.4%, $P = 0.016$) and longer hospital stay (14 d *vs* 13 d, $P = 0.011$). In the whole study group of 565 patients, 21 (3.7%) patients developed irreversible postoperative liver failure and 135 (23.9%) had hepatic insufficiency. Postoperative liver failure (6.8% *vs* 2.6%, $P = 0.02$) and hepatic insufficiency (31.5% *vs* 21.2%, $P < 0.001$) occurred more frequently in the Low Platelet Group. Postoperative mortality within 30 d was 2.1% (12 patients). Mortality in the Low Platelet Group was 6.8%, which was almost 14 times higher than that in the High Platelet Group with a mortality rate of 0.5% ($P < 0.001$). In addition, more patients in the Low Platelet Group had delayed recovery of liver function. Statistically significant differences were noted in alanine aminotransferase (ALT) levels on postoperative day 3 and day 5 (Figure 1), albumin level on postoperative day 5 (Figure 2) and total bilirubin on postoperative day 1, day 3 and day 5 (Figures 3 and 4). No significant differences were found in the other perioperative parameters.

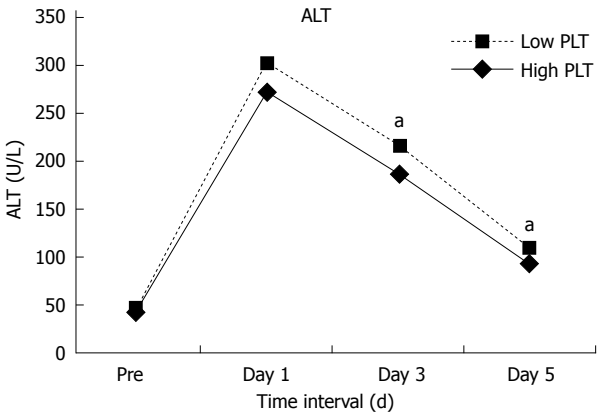

Figure 1 Alanine aminotransferase level in patients with low and high immediate postoperative platelet count. $^{a}P < 0.05$. ALT: Alanine aminotransferase; PLT: platelet count.

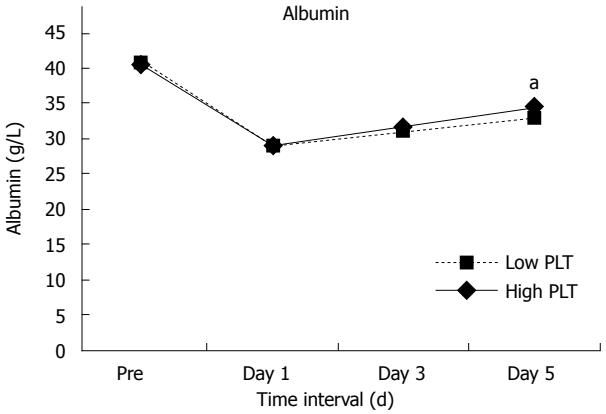

Figure 2 Albumin level in patients with low and high immediate postoperative platelet count. $^{a}P < 0.05$. PLT: platelet count.

Risk factors for delayed postoperative recovery of liver function

In order to identify the risk factors for postoperative hepatic insufficiency, a univariate analysis of patients with and without hepatic insufficiency was carried out. Univariate analysis (Table 3) showed that 11 variables were significantly associated with the occurrence of hepatic insufficiency. Both low preoperative platelet count and low postoperative platelet count were significant risk factors. The other 9 variables were male sex, esophageal varices, HBsAg, aspartate aminotransferase, ALT, total bilirubin, hemoglobin concentration, liver volume removed, and blood loss. These significantly different variables were included in a multivariate logistic regression model to identify whether low postoperative platelet count was an independent risk factor for hepatic insufficiency. The logistic regression analysis (Table 4) indicated that male sex, low postoperative platelet count, esophageal varices, total bilirubin, and liver volume removed were independent risk factors for hepatic insufficiency. Low platelet count remained a strong and independent risk factor for hepatic insufficiency, rather than low preoperative platelet count.

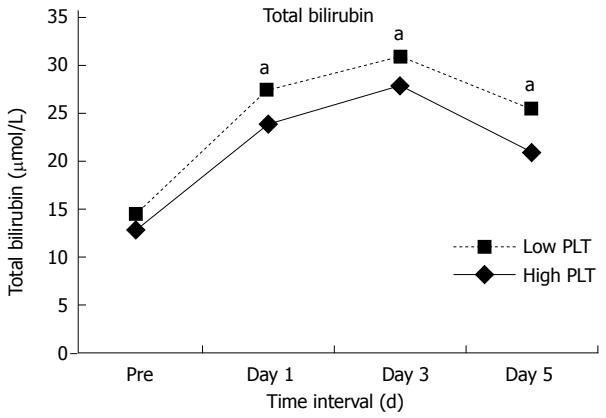

Figure 3 Bilirubin level in patients with low and high immediate postoperative platelet count. $^{a}P < 0.05$. PLT: platelet count.

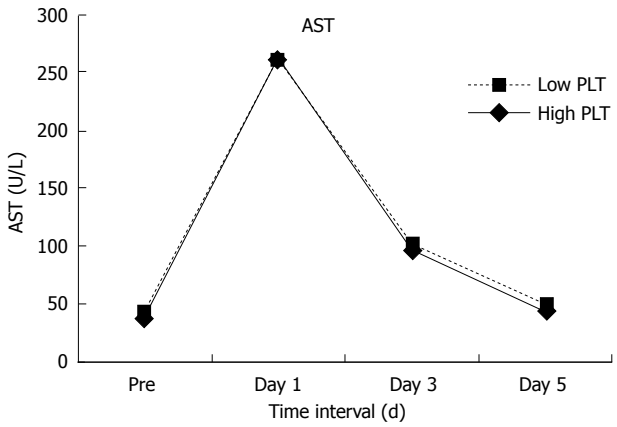

Figure 4 Aspartate aminotransferase level in patients with low and high immediate postoperative platelet count. AST: Aspartate aminotransferase; PLT: platelet count.

DISCUSSION

The perioperative outcomes after liver resection such as liver failure, hemorrhage and mortality are major concerns in hepatobiliary surgery, especially in HCC patients who usually have underlying liver diseases due to HBV infection. Many preoperative and intraoperative parameters affecting morbidity and mortality after hepatectomy have been evaluated, however, the effect of low postoperative platelet count on postoperative morbidity and mortality is not well known. We carried out a cohort study of 565 HCC patients undergoing major liver resection to assess whether immediate low postoperative platelet count affected postoperative outcomes. In our study, a low immediate postoperative platelet count was associated with liver failure, hepatic insufficiency and mortality in univariate and multivariate analyses. This demonstrated that low platelet count is an independent predictor of postoperative hepatic insufficiency.

To date, several studies have evaluated the role of platelets on morbidity and mortality. In addition, their short-term importance and positive significance have been confirmed. Three large high-volume studies[16-18] indicated that preoperative thrombocytopenia was as-

Table 3 Patient parameters in patients with or without liver dysfunction after major liver resection

Parameters	Liver dysfunction (*n* = 135)	No Liver dysfunction (*n* = 430)	OR (95%CI)	*P* value
Male	127 (94.1)	357 (83.0)	2.87 (1.42-5.79)	0.001
Age > 65 yr	12 (8.9)	62 (14.4)	0.58 (0.30-1.11)	0.097
Preoperative platelet < 100 × 10^9/L	37 (27.4)	70 (16.3)	1.94 (1.23-3.07)	0.004
Postoperative platelet < 100 × 10^9/L	46 (34.1)	100 (23.3)	1.71 (1.12-2.60)	0.012
BMI (kg/m^2), mean (SD)	22.9 (21.2-25.0)	22.5 (20.9-24.5)	Not available	0.147
Esophageal varices	36 (26.7)	33 (7.7)	4.38 (2.60-7.37)	0.001
Hypertension	22 (16.3)	74 (17.2)	0.94 (0.56-1.58)	0.805
Cardiovascular disease	4 (3.0)	11 (2.6)	1.16 (0.36-3.71)	0.799
Pulmonary disease	2 (1.5)	10 (2.3)	0.63 (0.14-2.92)	0.802
Diabetes mellitus	10 (7.4)	38 (8.8)	0.83 (0.4-1.70)	0.603
ASA Ⅲ-Ⅳ	17 (12.6)	62 (14.4)	0.86 (0.48-1.52)	0.594
HBsAg	119 (88.1)	335 (77.9)	2.11 (1.19-3.73)	0.009
HBeAg	24 (17.8)	56 (13.0)	1.44 (0.86-2.44)	0.167
HBV DNA > 2000 U/mL	47 (34.8)	139 (32.3)	1.12 (0.74-1.68)	0.591
AST (U/L) > ULN	75 (55.6)	187 (43.5)	1.62 (1.1-2.40)	0.014
ALT (U/L) > ULN	55 (40.7)	127 (29.5)	1.64 (1.10-2.50)	0.015
Total bilirubin (μmol/L), median (IQR)	16.1 (11.4-20.5)	12.5 (9.6-17.4)	Not available	< 0.001
Hemoglobin (g/L), median (IQR)	146 (131-157)	139 (127-150)	Not available	0.001
Liver volume removed				< 0.001
< 35%	15 (11.1)	145 (33.7)	1 (reference)	
35%-65%	32 (23.7)	97 (22.6)	3.19 (1.64-6.20)	
> 65%	88 (65.2)	188 (43.7)	4.53 (2.51-8.15)	
Extrahepatic procedures	37 (27.4)	86 (20.0)	1.51 (0.97-2.36)	0.069
Liver resection with				0.248
Hooking with ligation	38 (28.1)	100 (23.3)	1.29 (0.84-2.0)	
Ultrasonic dissection	97 (71.9)	330 (76.7)	0.77 (0.5-1.20)	
Inflow occlusion	54 (40.0)	197 (45.8)	1.27 (0.86-1.88)	0.236
Anatomic resection	98 (72.6)	289 (67.2)	1.29 (0.84-1.98)	0.240
Blood loss (mL), mean (SD)	770 (1049)	585 (433.0)	Not available	0.003
PRBCs transfusion	47 (34.8)	116 (27.0)	1.45 (0.96-2.19)	0.080
Hospital stay (d), median (IQR)	14 (11-19)	13 (11-16)	Not available	0.001

BMI: Body mass index; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen; SD: Standard deviation; ULN: Upper limit of normal; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; IQR: Interquartile range.

sociated with increased mortality or morbidity. Ishizawa *et al*[15] found that low platelet count was an independent risk factor for postoperative ascites. A study by Maithel *et al*[6] suggested that low preoperative platelet count may better serve with liver resection as a modified Child score which incorporates preoperative platelet count as a substitute for encephalopathy. However, with regard to the long-term effects of low platelet count, only one study has been carried out which suggested that the 3-year cumulative survival rate of HCC patients was comparable to those without thrombocytopenia[24]. Many studies have proved that a low preoperative platelet count was related to portal hypertension and its resulting hypersplenism and hepatic fibrosis[2,25]. Portal hypertension is considered a contraindication for liver resection because it significantly impairs liver function[2,7]. This is the reason why preoperative thrombocytopenia is associated with higher mortality and morbidity.

However, to our knowledge, few reports have been published on the effect of postoperative platelet count. Lesurtel[26] conducted a study in liver transplant patients with thrombocytopenia and found that a platelet count < 60 × 10^9/L on postoperative day 5 was an independent risk factor associated with severe complications and poor early graft and patient survival. Alkozai *et al*[19] reported a series of 216 patients with liver resection and demonstrated that a low immediate postoperative platelet count was an independent predictor of delayed postoperative liver function recovery and was associated with an increased risk of postoperative mortality. The study only included liver resection for colorectal liver metastases with normal liver parenchyma, however, the effect of low postoperative platelet count in HCC patients with underlying liver disease has not been studied.

The underlying mechanisms involved in the postoperative platelet count affecting postoperative liver function are not well understood. However, accumulating evidence from experimental and clinical studies indicated that platelets do not only have a role in hemostasis and thrombogenesis, but can also improve liver function by mediating liver regeneration[9]. Recent animal experiments suggested that platelets, or rather platelet-derived serotonin, contribute to cell cycle progression and metabolic pathways to prevent acute liver failure[9,11-13]. Other studies also proved that thrombopoietin[11] or platelets infused *via* the portal vein[10] can stimulate regeneration after hepatectomy in rats. This phenomenon in animal experiments was also confirmed in clinical practice. A retrospective study showed that transfused platelets were significantly associated with graft regeneration in liver donors[27].

Table 4 Multivariate analysis of independent risk factor for hepatic insufficiency

Variables	Regression coefficient (β)	OR (95%CI)	*P* value
Male	-0.993	0.393 (0.179-0.864)	0.020
Postoperative platelet < 100 × 10^9/L	0.597	1.816 (1.138-2.899)	0.012
Esophageal varices	1.446	4.244 (2.405-7.491)	< 0.001
Total bilirubin	0.072	1.075 (1.042-1.108)	< 0.001
Liver volume removed (%)		1 (Ref)	
35%-65%	1.185	3.271 (1.601-6.683)	0.001
> 65%	1.704	5.498 (2.908-10.394)	< 0.001

The role of platelets in the promotion of liver regeneration has been clinically confirmed in patients undergoing liver resection for colorectal liver metastases[19]. In Asia, in contrast to the liver with metastatic tumors, the liver with HCC usually has cirrhosis or fibrosis and secondary hypersplenism due to HBV infection. There is not only the well-known feature of thrombocytopenia, but also decreased platelet function in chronic HBV liver diseases and cirrhosis[8]. It is not known whether postoperative thrombocytopenia also has an effect on liver function recovery after liver resection for HBV-related HCC. Therefore, in the present study, we selected low immediate postoperative platelet count instead of preoperative platelet count as the criterion for thrombocytopenia, as platelet count usually changes due to intraoperative blood loss or platelet transfusion. In addition, preoperative platelet count appeared to be a surrogate for the preoperative severity of a patient's liver disease[26]. In our study, low immediate postoperative platelet count was associated with a greater likelihood of liver failure, hepatic insufficiency and mortality. Compared with patients with a high platelet count, the mortality rate was almost 14 times higher in patients with a low postoperative platelet count, and the rate of liver failure was 2.6 times higher. As there were a few cases involved, independent risk factors for liver failure and mortality were not analyzed by multivariate analysis. For hepatic insufficiency, multivariate analysis showed that liver volume removed was the strongest independent risk factor, followed by esophageal varices and platelet count. This differed from a previous study which found that low immediate postoperative platelet count was the strongest independent risk factor. A possible reasons for this difference was that patients in our study also had esophageal varices and underwent major liver resection. Portal hypertension (represented by esophageal varices) and major liver resection mainly contribute to hepatic insufficiency[5].

Our findings are instructive for surgeons to ensure that they take positive measures to increase platelet count to prevent hepatic insufficiency. These treatments should include platelet transfusion and administration of thrombopoietin and serotonin. One study[11] showed that administration of thrombopoietin reduces liver fibrosis and stimulates regeneration in the cirrhotic liver. This is suitable for HCC patients as most have different degrees of liver fibrosis which has a strong impact on morbidity. However, further research is needed before this treatment can be considered for use in the clinic.

Our study has several limitations mainly due to the retrospective analysis. It is important to point out that some of the patients in our study were highly selected for surgical safety. Secondly, the immediate postoperative platelet count may not be exact as it may have been affected by hemodilution or hemoconcentration after surgery. In addition, increased consumption of circulating platelets occurred due to subsequent bleeding and hemostasis after surgery. This was different in each patient and was not considered in our study.

In conclusion, our study found that a low immediate postoperative platelet count was associated with postoperative hepatic insufficiency, liver failure and mortality. A low immediate postoperative platelet count is an independent risk factor for hepatic insufficiency. These findings indicated that platelets can mediate liver regeneration in the cirrhotic liver.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is common and is one of the most common causes of cancer-related deaths in the world. Liver resection is performed as first-line treatment in patients with HCC. However, postoperative liver failure and hepatic insufficiency are common after liver resection, with the incidence rate ranging from 1.2% to 32%, which usually results in severe outcomes.

Research frontiers

Recent animal experiments suggested that platelets not only have a role in hemostasis and thrombogenesis, but can also improve liver function by mediating liver regeneration. Liver regeneration after liver resection can supply enough liver cells and increase the remnant liver volume to avoid liver failure and hepatic insufficiency. Several clinical studies have also indicated that a low preoperative platelet count is a risk factor associated with postoperative complications and mortality. In addition, a low immediate postoperative platelet count has been recently proved to be associated with delayed liver function recovery after partial liver resection for colorectal liver metastases.

Innovations and breakthroughs

This study found that patients with a low immediate postoperative platelet count < 100 × 10^9/L had more complications, and higher rates of postoperative liver failure, hepatic insufficiency and mortality. In addition, these patients had worse liver function after liver resection, with higher alanine aminotransferase, bilirubin and lower albumin levels.

Applications

A low immediate postoperative platelet count was an independent risk factor for hepatic insufficiency. These findings indicated that platelets can mediate liver regeneration in the cirrhotic liver.

Terminology

Platelets are non-nucleated discoid particles which are essential for hemostasis, liver regeneration and thrombosis. Low platelet count, also known as thrombocytopenia, is usually defined as platelets < 100 × 10^9/L, and is associated with poor hemostasis and liver regeneration.

Peer review

This paper brings the authors very interesting information about postoperative low platelet count associated with hepatic insufficiency after hepatectomy for hepatocellular carcinoma. The article is well redacted and its conclusions are very interesting for the international literature.

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RANDOMIZED CONTROLLED TRIAL

Influence of a probiotic mixture on antibiotic induced microbiota disturbances

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Abstract

AIM: To study the effect of probiotic consumption on the faecal microbiota during and after antibiotic exposure.

METHODS: A randomized, double-blind, placebo-controlled, parallel group study with a two species probiotic combination [*Lactobacillus acidophilus* (*L. acidophilus*) ATCC 700396 and *Bifidobacterium lactis* (*B. lactis*) ATCC SD5220] on healthy adults during and after antibiotic treatment (amoxicillin 875 and 125 mg clavulanate). The dominant faecal microbiota was studied by real time-polymerase chain reaction to determine if this probiotic preparation could facilitate restoring the microbiota to its pre-antibiotic state and influence the prevalence of beta-lactam resistance. Gastrointestinal symptoms were recorded by questionnaire and Bristol stool scale.

RESULTS: Subjects on the probiotic combination had significantly higher faecal counts of *L. acidophilus* ATCC 700396 and *B. lactis* at day 8 (end of antibiotic treatment period) *vs* those on placebo. Furthermore, subjects on the probiotic combination had significantly higher faecal counts of *L. acidophilus* ATCC 700396 and *B. lactis* at Day 15 (end of probiotic treatment) *vs* those on placebo. *Lactobacillus* counts remained stable in the probiotic group over the course of the study, while *Clostridium* XIV group was higher at the end of the study and closer to baseline levels; this in contrast to the placebo group. Beta-lactam resistance in creased after antibiotic exposure and was not different between both treatment groups. Gastrointestinal symptoms were generally mild and did not differ between the treatment groups, which correlates with the generally small changes in the microbiota.

CONCLUSION: Consumption of the probiotic combination mainly leads to an increase in the faecal levels of the species included in the preparation.

Key words: Probiotic; Antibiotic treatment; Amoxicillin/clavulanate; Microbiota; Beta-lactamases; *Lactobacillus acidophilus*; *Bifidobacterium lactis*

Core tip: The influence of a probiotic combination on the stability of the intestinal microbiota was studied using molecular techniques. Most published studies have relied on culturing or have only looked at symptomology. Furthermore, this was studied in a antibiotic challenge setting to limit variability.

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INTRODUCTION

Although the antimicrobial properties of antibiotics have provided great medical benefits, they may also affect the composition and activity of, in particular, the intestinal microbiota. This disturbance in the balance and diversity of the composition of the normal intestinal microbiota has been identified as the major factor involved in the pathogenesis of antibiotic associated diarrhoea (AAD)[1]. The magnitude of these changes is influenced by the dose, type and duration of antibiotic use, along with the capability of the intestinal microbiota to resist colonization changes.

Treatment possibilities for AAD are limited, but probiotics have been suggested as a potential way to counteract the potential negative effects of antibiotics. The Food and Agricultural Organization of the United Nations and World Health Organization have defined probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host"[2]. Various strains of probiotics have been shown to protect against bacterial and viral enteropathogens by producing inhibitory antimicrobial substances such as organic acids, hydrogen peroxide and bacteriocins, and demonstrating competitive inhibition for bacterial adhesion sites on intestinal epithelial surfaces[3]. Such properties may make probiotics good candidates for stabilizing the intestinal microbiota during antibiotic challenge. Selected probiotic preparations have been shown to reduce antibiotic induced microbiota disturbances[4]. Furthermore, many strains of probiotics have also been found to reduce the incidence of AAD; a recent meta-analysis concluded that probiotics are associated with a reduced risk for AAD[5].

The primary objective of the present study was to investigate the effect of a specific combination of probiotic strains on the incidence of antibiotic induced microbiota disturbances. The secondary objectives were to investigate the influence of probiotics on quality of life and stool consistency during and following antibiotic use.

MATERIALS AND METHODS

The study was reviewed and approved by the Therapeutic Products Directorate, in consultation with the Natural Health Products Directorate, Health Canada, and Institutional Review Board Services (Aurora, ON, Canada), and conducted in accordance with the Declaration of Helsinki.

Study design

The study was triple-blind, randomized, placebo controlled with two parallel study groups. Participants were stratified by gender at a ratio of 1:1. After successful screening, all volunteers received amoxicillin and clavulanate daily from day 1 to 7 and were randomly allocated to receive either probiotic or placebo daily from day 1 to 14, where after the volunteers had a 7 d follow up period.

For the study, 111 participants were screened; 80 were enrolled (Figure 1). The inclusion criteria were male or female aged 18 to 50 years; if female, either not of child bearing potential or using a medically approved method of birth control; body mass index 18.0: 29.9 kg/m^2; healthy as determined by laboratory results, medical history and physical exam; agreed not to change current dietary habits (with the exception of avoiding pro- and prebiotics) and activity/training levels during the course of the study; gave voluntary, written, informed consent to participate in the study. Exclusion criteria were - women who were pregnant, breastfeeding, or planning to become pregnant during the course of the trial; body mass index $\geqslant$ 30 kg/m^2; average number of formed bowel movements > 3 per day or < 3 per week; smokers (ex-smokers must have quit at least 3 mo prior); participation in a clinical research trial within 30 d prior to randomization; use of antibiotics within 60 d prior to randomization; habitual use of pro- and/or prebiotic products; followed a vegetarian or vegan diet; unstable medical conditions; history of chronic gastrointestinal disorders; alcohol use > 2 standard alcoholic drinks per day and/or alcohol or drug abuse within past year; allergy or sensitivity to test product ingredients or antibiotic (amoxicillin and clavulanate), allergy to any penicillin antibiotic or cephalosporin antibiotic; individuals who were cognitively impaired and/or unable to give informed consent; any other condition which, in the investigator's opinion, may adversely affect the subject's ability to complete the study or its measures or which may pose significant risk to the subject.

Study products

The study products consisted of 12.5 × 10^9 CFU/d *Lactobacillus acidophilus* (*L. acidophilus*) ATCC 700396 and 12.5 × 10^9 CFU/d *Bifidobacterium animalis* (*B. animalis*) ssp. *lactis* ATCC SD5220 (Danisco USA, Madison, WI, United States) in a hypromellose capsule. Maltodextrin was used as an excipient. The placebo consisted of the same capsule with only maltodextrin. At the end of the study, viable counts were determined and found to have deviated less than 10% from the target count.

The antibiotic used was Augmentin (Apotex, Toronto, Canada); 875 mg amoxicillin and 125 mg clavulanate.

Compliance

Compliance was assessed by counting the returned study product and antibiotic at each visit. Compliance was calculated as a percentage by determining the number of dosage units consumed divided by the number expected to have been taken multiplied by 100%. In the event of a discrepancy between the information in the subject diary and the amount of study product returned, calculations were based on the product returned unless an explanation for loss of product was provided. Participants found to have a compliance of < 80% or > 120% at any visit were counselled. A compliance of < 70% or > 130% was considered as non-compliant and any subject demonstrating non-compliance for two consecutive visits was to be withdrawn from the study. Compliance rates over 100% were explained by a visit later then intended and

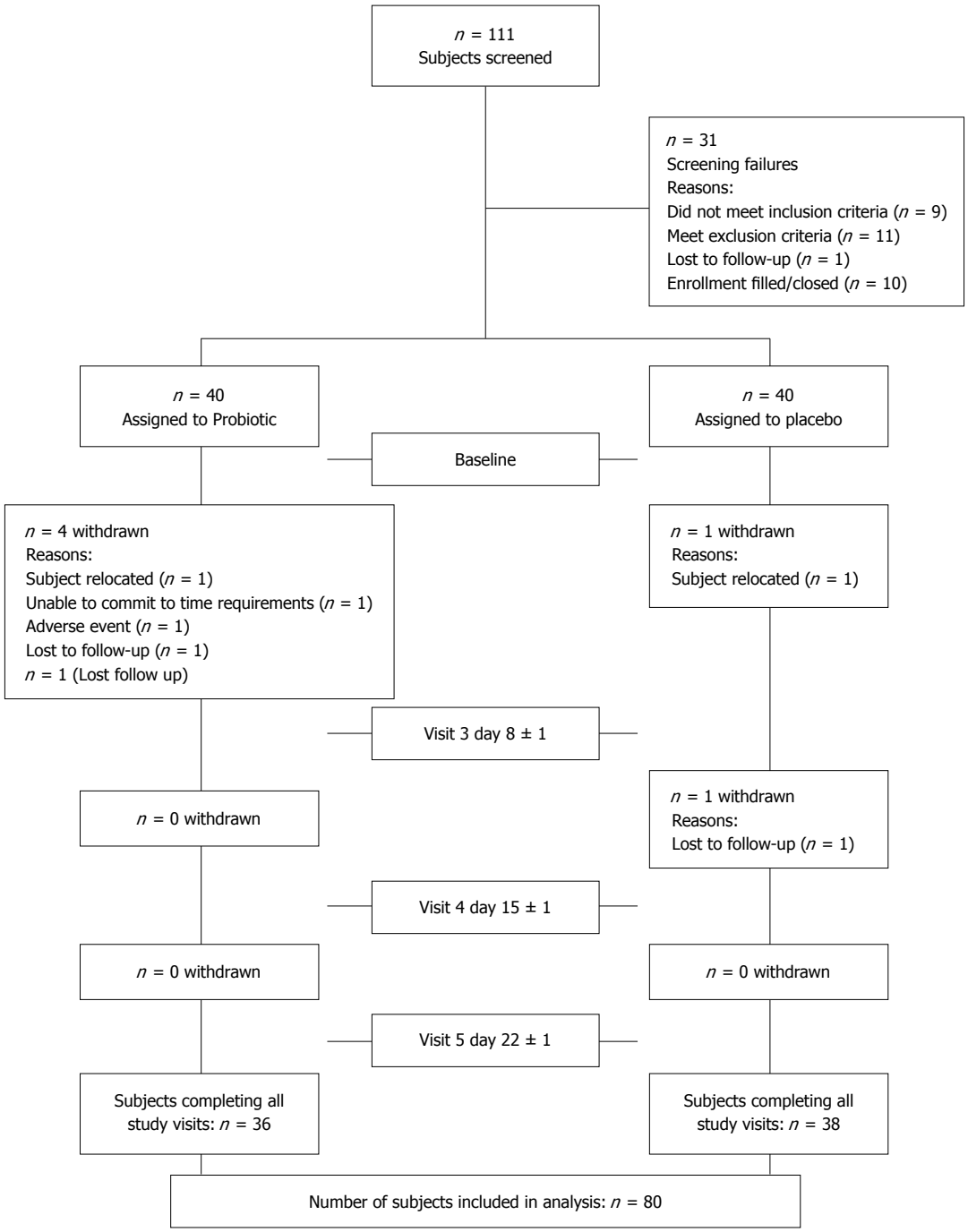

Figure 1 CONSORT patient flow diagram.

additional consumption of study product.

Outcomes

The primary objective was to evaluate the maintenance of the intestinal microbiota composition during antibiotic (amoxicillin and clavulanate) treatment by quantitative real-time polymerase chain reaction (qPCR). To this end, the following commensal and potential pathogenic microbial groups were analysed from the faecal samples: *Lactobacillus* spp.[6], *L. acidophilus* ATCC 700396[7], *Bifidobacterium*[8], *B. lactis*[9,10], *Bacteroides*[11], *C. difficile*[6], *Clostridium* cluster XIV[12] and *Enterobacteriaceae*[13] by qPCR; using a ABI 7500 FAST sequencing detection system (Applied Biosystems Foster City, United States). Ten-fold dilution series (10 pg and 1 ng) of DNA from the standard strains were used for the standard curves. For the determination of DNA, triplicates of each sample were run, and the mean quantity per gram faecal wet weight was calculated. The total bacterial count was analyzed by flow cytometry as described previously[14].

Prevalence of antibiotic resistance caused by the extended-spectrum beta-lactamases (ESBL) was analyzed by a PCR and hybridisation combined method using a commercial Multiplex ESBL kit (BIORON Diagnostics

Table 1 Demographic description of the enrolled volunteers *n* (%)

	Probiotic (n = 40)	Placebo (n = 40)	P value
Female	20 (50)	20 (50)	
Male	20 (50)	20 (50)	
Age (yr)	33.7 ± 9.4	30.9 ± 10.3	0.164
Weight (kg)	72.5 ± 12.9	71.5 ± 12.1	0.706
Height (cm)	171.2 ± 8.7	170.5 ± 10.3	0.766
BMI (kg/m²)	24.7 ± 3.5	24.5 ± 2.7	0.743
Hispani or Latino	4 (10)	6 (16)	
African American	3 (7)	1 (2)	
White	29 (73)	32 (80)	
Other	4 (10)	1 (2)	
Alcohol use			
None	8 (20)	14 (35)	0.013
Occasionally	28 (70)	15 (38)	
Weekly	4 (10)	11 (28)	
Ex-smoker	2 (5)	4 (10)	
n	38 (95)	36 (90)	

BMI: Body mass index.

GmbH, Ludwigshafen, Germany). This kit detects a selection of potentially ESBL-positive bacteria by detecting all variants of the genes bla$_{TEM}$, bla$_{SHV}$, bla$_{CTX-M}$ and relevant ESBL phenotypic variants of bla$_{OXA}$.

Secondary outcomes consisted of Gastrointestinal Symptom Rating Scale (GSRS), bowel habit scores; frequency and consistency (Bristol Stool scale) and adverse events. The GSRS is a disease-specific questionnaire of 15 items combined into five symptom clusters depicting reflux, abdominal pain, indigestion, diarrhoea and constipation; with a scale from 1 to 7 for no to serious symptoms. The GSRS is well-documented[15] and norm values for a general population have been established[16]. Bowel habits were scored on a diary; for number of bowel movements, straining to start defecation, straining to stop defecation, feeling of incomplete defecation and use of laxatives. Finally, stool form was scored according to the Bristol Stool scale which describes and depicts the form of the faeces on a 7 point scale, from hard (1) to watery (7)[17].

Adverse events, especially those for which the relationship to investigational product was suspected, were to be recorded and followed up on until they returned to baseline status or stabilized. In the rare event of microbial overgrowth with subject displaying the symptoms of a bacterial infection, the study physician was instructed to prescribe an antibiotic to which the strains were known to be susceptible.

Sample size

A per group sample size of 40 participants was required to detected a clinically significant difference of 10% at 80% power, a = 0.05 (2-sided), 15% difference in statistical methods, allowing for a 20% attrition rate[18].

Randomization

A randomization schedule was created by the manufacturer. Participants were stratified by gender. A total of 112 randomizations were provided (56 males and 56 females), to account for additional recruitment, lost bottles, etc. The unique randomization numbers (112) were created using randomizer.org for each test product and divided over 28 blocks of 4.

Statistical analysis

Data was analyzed on the basis of intention-to-treat. Frequency counts and proportions were used to describe categorical variables. Subject demographics were compared between groups using unpaired Student t test, Fisher exact test or χ^2 test as appropriate. For outcomes with continuous variables, comparisons of changes over time were analyzed by paired Student t test of Wilcoxon signed-rank test. Differences between treatments were analyzed by unpaired t test or Mann-Whitney U test.

RESULTS

Study participants and demographics

Subject demographics and characteristics were similar for both treatment groups, with the exception of alcohol use (P = 0.013), where the probiotic group tended to have more occasional drinkers (Table 1). Two participants in the placebo group and four participants in the probiotic group did not complete the study (Figure 1).

Compliance

Compliance of antibiotics use was greater than 99% (standard deviation 2.7%) in both treatment groups. Compliance of probiotic/placebo use was greater than 100% (standard deviation 5.5% for the first week and 9.4% for the second week).

Microbiota composition

At baseline, no differences were detected between the two groups for any of the tested microbial taxa (Table 2).

Subjects randomized to receive probiotics had increased faecal counts of *L. acidophilus* ATCC 700396 at the end of antibiotic treatment period and at the end of study product treatment period compared to those receiving placebo (Table 2). When comparing between groups, the probiotic group had significantly higher levels of *B. lactis* and *L. acidophilus* ATCC 700396 than the placebo group as long as the study products were consumed. *Lactobacillus* levels were not affected by the antibiotic in the probiotic group; this in contrast to the placebo group. Furthermore, the *B. lactis* levels were restored to base line after completing probiotic consumption. In the placebo group, *B. lactis* levels were still not restored to baseline at the end of the study (Table 2).

Within groups, after one week of antibiotic and probiotic or placebo consumption, total bacterial counts and *Clostridium* cluster XIV counts both decreased from baseline. On the other hand, *Enterobacteriaceae* were significantly increased in both groups (P < 0.001). In the placebo group, *Lactobacillus* spp. levels and *B. lactis* levels were reduced compared to baseline, while in the probiotic

Table 2 Bacterial counts

	Probiotic	*P* value (within group)	Placebo	*P* value	
				Within group	Between group
Total bacteria					
Baseline	10.89 ± 0.22	-	10.81 ± 0.23	-	0.067
End of antibiotic + probiotic/placebo	10.58 ± 0.43	< 0.001	10.49 ± 0.38	< 0.001	0.177
End of probiotic/placebo	10.75 ± 0.30	0.003	10.77 ± 0.26	0.399	0.735
End of follow-up	10.87 ± 0.24	0.527	10.79 ± 0.31	0.568	0.221
Lactobacillus					
Baseline	7.40 ± 0.79	-	7.42 ± 1.56	-	0.391
End of antibiotic + probiotic/placebo	7.13 ± 0.88	0.104	6.91 ± 1.45	0.032	0.642
End of probiotic/placebo	7.42 ± 0.77	0.944	7.07 ± 1.38	0.030	0.331
End of follow-up	7.16 ± 1.50	0.375	6.96 ± 1.87	0.149	0.851
Lactobacillus acidophilus ATCC 700396					
Baseline	1.27 ± 2.20	-	0.93 ± 1.73	-	0.446
End of antibiotic + probiotic/placebo	2.39 ± 2.98	0.052	1.21 ± 2.07	0.407	0.035
End of probiotic/placebo	2.15 ± 2.79	0.245	0.79 ± 1.54	0.933	0.011
End of follow-up	1.60 ± 2.51	0.286	2.13 ± 2.74	0.021	0.498
Bifidobacterium					
Baseline	8.62 ± 1.60	-	7.97 ± 1.99	-	0.087
End of antibiotic + probiotic/placebo	8.20 ± 1.08	0.016	7.83 ± 1.78	0.350	0.642
End of probiotic/placebo	8.72 ± 0.79	0.759	8.01 ± 2.13	0.805	0.142
End of follow-up	8.52 ± 1.60	0.466	8.12 ± 1.57	0.422	0.236
Bifidobacterium lactis					
Baseline	8.81 ± 0.50	-	8.82 ± 0.69	-	0.914
End of antibiotic + probiotic/placebo	8.41 ± 1.48	0.054	8.20 ± 0.64	< 0.001	0.008
End of probiotic/placebo	8.79 ± 0.63	0.904	8.42 ± 0.67	< 0.001	0.013
End of follow-up	8.67 ± 0.49	0.206	8.49 ± 0.74	< 0.001	0.185
Bacteroides					
Baseline	9.14 ± 0.56	-	8.97 ± 0.48	-	0.161
End of antibiotic + probiotic/placebo	9.06 ± 0.87	0.981	9.00 ± 0.61	0.629	0.345
End of probiotic/placebo	8.98 ± 0.69	0.050	8.85 ± 0.63	0.194	0.429
End of follow-up	9.14 ± 0.63	0.972	8.91 ± 0.54	0.479	0.079
Enterobacteriaceae					
Baseline	6.92 ± 0.83	-	6.74 ± 1.33	-	0.734
End of antibiotic + probiotic/placebo	7.80 ± 1.16	< 0.001	7.68 ± 1.07	< 0.001	0.531
End of probiotic/placebo	6.88 ± 0.73	0.944	6.87 ± 0.72	0.732	0.947
End of follow-up	6.89 ± 0.55	0.956	6.75 ± 1.33	0.553	0.770
Clostridium difficile					
Baseline	2.85 ± 1.44	-	2.90 ± 1.29	-	0.563
End of antibiotic + probiotic/placebo	3.42 ± 2.30	0.283	2.95 ± 2.54	0.632	0.281
End of probiotic/placebo	3.13 ± 1.42	0.566	2.56 ± 1.76	0.126	0.077
End of follow-up	3.08 ± 1.44	0.712	2.91 ± 1.68	0.475	0.935
Clostridium group XIV					
Baseline	10.04 ± 0.24	-	9.92 ± 0.39	-	0.073
End of antibiotic + probiotic/placebo	9.36 ± 0.72	< 0.001	9.36 ± 0.45	< 0.001	0.582
End of probiotic/placebo	9.85 ± 0.32	< 0.001	9.74 ± 0.37	0.004	0.146
End of follow-up	9.94 ± 0.25	0.046	9.75 ± 0.36	0.006	0.011

Bacterial counts (log10 counts/g wet weight) at baseline (day 1), after 1 wk treatment period with antibiotic + probiotic or placebo (day 8), after 1 wk of supplementation with probiotic or placebo only (day 15) and after 1 wk follow-up period (day 22). Data are expressed as mean ± SD.

group, *Bifidobacterium* spp. levels were decreased. The level of *L. acidophilus* ATCC 700396 was increased compared to baseline (Table 2).

After the additional week on probiotic or placebo, without antibiotics, *Clostridium* cluster XIV levels remained significantly reduced in both groups when compared to baseline. In the placebo group, *Lactobacillus* levels and *B. lactis* levels remained below baseline. In the probiotic group, *Lactobacillus* levels and *B. lactis* levels were restored to base-line but total bacterial numbers remained and *Bacteroides* remained below baseline (Table 2).

After follow up, which was the last week of the study where volunteers did not receive either probiotic or placebo. *Clostridium* cluster XIV levels remained reduced in both groups when compared to baseline. In the placebo group, levels of *L. acidophilus* ATCC 700396 increased to above baseline levels while *B. lactis* remained below baseline (Table 2).

Prevalence of antibiotic resistance

One subject within each group had a positive baseline sample for beta-lactam resistance. After antibiotic treatment, 16 participants in the probiotic group and 14 participants in the placebo group showed a positive signal for beta-lactam resistance ($P = 0.924$). None of the samples were positive for ESBL production.

Table 3 Gastrointestinal Symptom Rating Scale scores for the study participants

		Probiotic		Placebo		
		(*n* = 40)	*P* value (within group)	(*n* = 40)	*P* value	
					Within group	Within group
Stomach ache or pain	Baseline	1.10 ± 0.38	-	1.32 ± 0.73	-	0.101[1]
	End of antibiotic	1.82 ± 1.32	0.002[1]	1.80 ± 1.26	0.032[1]	0.991[1]
	End of treatment	1.48 ± 1.22	0.103[1]	1.45 ± 1.01	0.632[1]	0.829[1]
	End of study	1.38 ± 0.98	0.131[1]	1.48 ± 1.15	0.671[1]	0.741[1]
Nausea	Baseline	1.00 ± 0.00	-	1.23 ± 0.66	-	0.022[1]
	End of antibiotic	1.50 ± 1.26	0.022[1]	1.60 ± 1.15	0.088[1]	0.360[1]
	End of treatment	1.27 ± 0.93	0.054[1]	1.15 ± 0.36	0.608[1]	0.828[1]
	End of study	1.12 ± 0.79	> 0.999[1]	1.15 ± 0.53	0.608[1]	0.187[1]
Rumbling in stomach	Baseline	1.55 ± 0.75	-	1.50 ± 0.75	-	0.743[1]
	End of antibiotic	1.95 ± 1.34	0.038[1]	1.80 ± 1.36	0.187[1]	0.234[1]
	End of treatment	1.73 ± 1.26	0.565[1]	1.42 ± 0.87	0.488[1]	0.278[1]
	End of study	1.50 ± 0.93	0.388[1]	1.35 ± 0.77	0.179[1]	0.267[1]
Bloated	Baseline	1.25 ± 0.44	-	1.30 ± 0.76	-	0.547[1]
	End of antibiotic	1.85 ± 1.48	0.010[1]	1.32 ± 0.76	0.936[1]	0.071[1]
	End of treatment	1.50 ± 0.96	0.197[1]	1.32 ± 0.80	> 0.999[1]	0.242[1]
	End of study	1.50 ± 0.88	0.096[1]	1.40 ± 1.08	0.719[1]	0.296[1]
Flatulus	Baseline	1.57 ± 0.81	-	1.52 ± 0.78	-	0.834[1]
	End of antibiotic	2.00 ± 1.36	0.029[1]	1.62 ± 0.98	0.857[1]	0.239[1]
	End of treatment	1.70 ± 1.30	> 0.999[1]	1.45 ± 0.99	0.331[1]	0.303[1]
	End of study	1.52 ± 1.13	0.291[1]	1.45 ± 0.85	0.492[1]	0.689[1]
Diarrhea	Baseline	1.18 ± 0.55	-	1.20 ± 0.79	-	0.512[1]
	End of antibiotic	1.92 ± 1.53	0.001[1]	1.73 ± 1.26	0.050[1]	0.614[1]
	End of treatment	1.45 ± 1.28	0.260[1]	1.20 ± 0.61	> 0.999[1]	0.670[1]
	End of study	1.35 ± 1.05	0.389[1]	1.15 ± 0.70	0.892[1]	0.094[1]
Loose stools	Baseline	1.25 ± 0.63	-	1.25 ± 0.71	-	0.793[1]
	End of antibiotic	1.70 ± 1.07	0.002[1]	1.48 ± 0.75	0.109[1]	0.492[1]
	End of treatment	1.48 ± 0.99	0.241[1]	1.20 ± 0.46	0.784[1]	0.322[1]
	End of study	1.25 ± 0.44	0.824[1]	1.27 ± 0.75	> 0.999[1]	0.503[1]
Bowel movement	Baseline	1.25 ± 0.67	-	1.40 ± 0.84	-	0.291[1]
	End of antibiotic	1.73 ± 1.20	0.032[1]	1.73 ± 1.06	0.116[1]	0.739[1]
	End of treatment	1.52 ± 1.13	0.208[1]	1.35 ± 0.77	0.813[1]	0.712[1]
	End of study	1.38 ± 1.10	0.717[1]	1.32 ± 0.86	0.565[1]	0.816[1]
Acid reflux	Baseline	1.55 ± 0.71	-	1.55 ± 0.64	-	0.850[1]
	End of antibiotic	1.60 ± 0.90	0.805[1]	1.55 ± 0.81	0.960[1]	0.890[1]
	End of treatment	1.52 ± 0.72	0.894[1]	1.35 ± 0.66	0.129[1]	0.210[1]
	End of study	1.27 ± 0.60	0.034[1]	1.35 ± 0.66	0.162[1]	0.496[1]
Constipation	Baseline	1.27 ± 0.51	-	1.25 ± 0.59	-	0.517[1]
	End of antibiotic	1.52 ± 1.15	0.266[1]	1.65 ± 1.25	0.007[1]	0.625[1]
	End of treatment	1.42 ± 1.11	0.822[1]	1.30 ± 0.76	0.857[1]	0.605[1]
	End of study	1.60 ± 1.24	0.108[1]	1.65 ± 1.25	0.034[1]	0.708[1]
Overal GSRS	Baseline	1.272 ± 0.280	-	1.322 ± 0.319	-	0.444[1]
	End of antibiotic	1.60 ± 0.76	< 0.001[1]	1.54 ± 0.58	0.007[1]	0.969[1]
	End of treatment	1.45 ± 0.79	0.509[1]	1.28 ± 0.33	0.192[1]	0.481[1]
	End of study	1.36 ± 0.70	0.740[1]	1.32 ± 0.57	0.264[1]	0.864[1]

Data are expressed as mean ± SD. [1]After a *P* value indicates that it was obtained from a non-parametric test, such as the Wilcoxon or Mann-Whitney *U* test. This is done whenever the values being summarized are significantly non-normally distributed, as assessed by the Anderson-Darling test. GSRS: Gastrointestinal Symptom Rating Scale.

GSRS

In general, GSRS scores were low; 2 or less; *i.e.*, no or slight discomfort. Nausea was reported more in the placebo group at baseline compared to the probiotic group (Table 3). No other differences were reported between groups at baseline.

Following antibiotic consumption, both groups reported increased stomach ache or pain, nausea and diarrhoea; the numbers were similar between the groups and normalized in the following weeks (Table 3). Though not significantly different between the groups, the probiotic group reported a reduction in acid reflux after the follow up week (Table 3). On the other hand, participants in the placebo group reported more constipation after the antibiotic and placebo week ($P = 0.007$) and after the follow up week ($P = 0.034$).

Over all, total GSRS scores were different for both groups only after the week with antibiotics; probiotic ($P < 0.001$) and placebo ($P = 0.007$) group. There was no difference between groups for the overall GSRS score at any of the assessed time points.

Bowel habits

Although volunteers in the probiotic group had a sig-

nificant increase in bowel movements after antibiotic administration ($P = 0.032$) this was not different from the placebo group.

Bristol stool scale

Subjects in both groups reported increased Bristol stool scale values with the highest stool scale value on day three of the antibiotic period. The probiotic group tended to have somewhat looser stools then the placebo group.

Adverse events

A total of 59 adverse events were reported during the study by 35 participants. All adverse events resolved before the end of study. There was no significant difference in the number of participants reporting any adverse event between treatment groups; 16 in the probiotic group and 19 in the placebo group. In the probiotic group, one subject withdrew during the antibiotic supplementation period due to upset stomach (Figure 1). No serious adverse events were reported during the study.

DISCUSSION

Antibiotics have brought great benefits to medical practice. However, their antimicrobial activities affect not just the targeted pathogen, but also the endogenous microbiota of the host. This disturbance in microbiota composition and activity is considered to be one of the reasons for AAD[1,19]. Most studies on AAD and probiotics use patients as their study population. However, the use of patients introduces variability as the participants have different underlying diseases and usually get prescribed various antibiotics for various lengths of time and at different doses. When studying the effect of antibiotics on the intestinal microbiota and how probiotics may influence this, patients are not usually able to provide a baseline sample. The design of the current study, using healthy volunteers that took the same antibiotic for the same length of time, allowed the baseline to be established and eliminated variation that may have resulted from differing lengths and doses of antibiotic usage. The study design does, however, not allow for conclusions on other antibiotic regimens and/or probiotic preparations. A similar study set up indicated that a combination of five probiotic strains was able to maintain the overall intestinal microbiota composition[4]. However, the study did not investigate specific microbial groups and the consumed probiotic strains by molecular methods, as was done in the present study.

The antibiotic induced limited changes in the faecal microbiota. The changes that were observed, were small and although statistically significant, the biological relevance may be limited. Total bacterial numbers (by faecal wet weight) were reduced in both treatment groups, which can be explained by the looser stools that were produced. The reduction in lactobacilli in the placebo group was not observed in the probiotic group and may be explained by the consumption of the probiotic that contained a *Lactobacillus* and may suggest a stabilisation of the faecal *Lactobacillus* levels by the probiotic. Likewise, levels of *L. acidophilus* ATCC 700396 and *B. lactis* were higher or more stable in the probiotic group; which was also likely related to the consumption of these strains/species. The apparent increase in *L. acidophilus* ATCC 700396 levels in the placebo group at the end of the follow up period can be explained by the inadvertent consumption of probiotic products by some volunteers. *Enterobacteriaceae* were increased in both groups after the antibiotic consumption and this was not influenced by the consumption of probiotics. *C. difficile* was not influenced by either the antibiotic or the probiotic, which was contrary to earlier observations where *L. acidophilus* ATCC 700396, together with *L. rhamnosus* HN001 was able to reduce the level and number of participants carrying *C. difficile*[6].

Only broad-spectrum beta-lactamases could be detected; mainly after the antibiotic exposure, and there was no difference in prevalence between the two groups. Thus, the probiotics did not influence the emergence of beta-lactamase in the microbiota. None of the analyzed samples were positive for ESBL. The participants within this study were healthy adults, and since ESBLs are mostly prevalent in nosocomial settings[20], this may explain the absence of ESBLs.

The limited disturbance of the faecal microbiota correlates well with the limited gastrointestinal complaints reported by the volunteers. While a significant increase in various symptoms was reported; these did not exceed a level of slight discomfort; Bristol stool scale values remained in the normal range and the number of passed stools did not reach the level defined for diarrhoea which is 3 or more loose stools per day. The general mild symptoms could be explained by a relatively short exposure and low dose of antibiotics.

In conclusion, consumption of amoxicillin and clavulanate by healthy volunteers caused only minimal microbiota disturbances. Probiotic consumption lead only to small increased faecal levels of the consumed genera and species.

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COMMENTS

Background

Antibiotics have the potential to disturb the intestinal microbiota. This disturbance is one of the causes of antibiotic associated diarrhea (AAD). Probiotics have been shown to reduce the risk of AAD. The mechanism is thought to be by stabilisation of the microbiota, but this has been little investigated.

Research frontiers

Studying antibiotic induced changes in the faecal microbiota composition have

been investigated only to a limited extent with molecular techniques as has the effect of probiotics on the microbiota.

Innovations and breakthroughs

To study the effect of probiotics on antibiotic induced changes in the faecal microbiota, a challenge model was used where healthy volunteers under defined conditions were exposed to antibiotics and probiotics or placebo in a randomised and blinded study set up.

Applications

Probiotics have been documented to reduce the risk for AAD. However, contrary to the common perception, the tested antibiotic (amoxicillin-clavulanate) appeared to cause only limited disturbance of the intestinal microbiota and hence the effect of probiotics on this was limited. Probiotics may therefore work through a different mechanism on AAD. The administered species are found to be increased in the faeces.

Terminology

Probiotic: live microorganisms which when administered in adequate amounts confer a health benefit on the host. Microbiota: the microflora (and microfauna) in an ecosystem (usually an animal host or a single part of its body, such as intestines, mouth, vagina, *etc.*). Antibiotic associated diarrhoea results from an imbalance in the colonic microbiota caused by antibiotic therapy causing an osmotic diarrhea or allowing the overgrowth of potentially pathogenic organisms.

Peer review

It may be worth to be published because of all the uncertainties around the use of probiotics to prevent gastrointestinal disorders related to antibiotic treatments.

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META-ANALYSIS

S-1-based *vs* non-S-1-based chemotherapy in advanced gastric cancer: A meta-analysis

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Abstract

AIM: To assess the efficacy and tolerability of S-1-based *vs* non-S-1-based chemotherapy in advanced gastric cancer (AGC).

METHODS: We extracted reported endpoints, including overall survival (OS), progression-free survival (PFS), time-to-treatment failure (TTF), objective response rate (ORR) and adverse effects, from randomized controlled trials identified in PubMed, the Cochrane library, Science Direct, EMBASE and American Society of Clinical Oncology meetings. Stata software was used to calculate the pooled values.

RESULTS: Seven randomized controlled trials involving 2176 patients were included in this meta-analysis. Compared to non-S-1-based regimens, the use of S-1-based regimens were associated with an increase in ORR (RR = 1.300; 95%CI: 1.028-1.645); OS (HR = 0.89; 95%CI: 0.81-0.99; P = 0.025), TTF (HR = 0.83; 95%CI: 0.75-0.92; P = 0.000), and a lower risk of febrile neutropenia (RR = 0.225; P = 0.000) and stomatitis (RR = 0.230; P = 0.032). OS, PFS and TTF were prolonged, especially in the Asian population. In subgroup analysis, statistically significant increases in ORR (RR = 1.454; P = 0.029), OS (HR = 0.895; P = 0.041) and TTF (HR = 0.832; P = 0.000) were found when S-1-based chemotherapy was compared to 5-fluorouracil (5-FU)-based chemotherapy. The incidence of leukopenia (RR = 0.584; P = 0.002) and stomatitis (RR = 0.230; P = 0.032) was higher in the 5-FU-based arm. S-1-based regimens had no advantage in ORR, OS, PFS, TTF and grade 3 or 4 adverse events over capecitabine-based regimens.

CONCLUSION: S-1-based chemotherapy may be a good choice for AGC because of longer survival times, better tolerance and more convenient use.

Key words: S-1; Advanced gastric cancer; Chemotherapy; First line treatment; Meta-analysis

Core tip: This meta-analysis aimed to assess the efficacy and tolerability of S-1-based *vs* non-S-1-based chemotherapy in advanced gastric cancer (AGC). Compared to non-S-1-based regimens, the use of S-1-based regimens were associated with an increase in the objective response rate, overall survival, time-to-treatment failure, and a lower risk of grade 3 or 4 adverse events. S-1-based chemotherapy may be a good choice for AGC, at least in Asia because of longer survival times, better tolerance and more convenient use.

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INTRODUCTION

Although gastric cancer rates have decreased substantially in most parts of the world[1] because of advances in early diagnosis, control of chronic *Helicobacter pylori* infection and changes in lifestyles, it remains a common and devastating disease. A total of 989600 new gastric cancer cases and 738000 deaths are estimated to have occurred in 2008, accounting for 8% of the total cases and 10% of total deaths[2]. Nowadays, surgery remains the primary treatment, with an average 5-year survival rate of 20%-30%. More than two-thirds of patients have unresectable disease when diagnosed[3], so chemotherapy is regarded as a significant and basic treatment method. Compared with the best supportive care, chemotherapy increases the 1-year survival rate and provides a longer symptom-free period of 6 mo and an improvement in quality of life[4,5]. Many studies based on combinations of new-generation agents, like S-1, capecitabine, taxanes, oxaliplatin and irinotecan have been undertaken[5-8], and new and more effective regimens are being explored.

S-1 is a novel oral derivative of 5-FU, and contains tegafur/gimeracil/oteracil potassium in a molar ratio of 1.0:0.4:1.0. Tegafur (FT) is a depot form of fluorouracil, which releases 5-fluorouracil (5-FU) slowly in the body[9]. Gimeracil, a dihydropyrimidine dehydrogenase inhibitor, contributes to a decrease in 5-FU catabolism and to significantly higher blood levels of 5-FU compared to FT alone[10,11]. Oteracil potassium (Oxo), another enzyme inhibitor of 5-FU, can suppress the gastrointestinal toxicity of FT[12]. In theory, S-1 is more tolerable and effective than 5-FU, and will be more convenient to use for patients with advanced gastric cancer (AGC). Based on the encouraging results from a number of phase II trials for S-1-based chemotherapy[13-19], some randomized controlled trials were carried out to compare S-1-based chemotherapy and non-S-1-based chemotherapy. However, there is controversy and uncertainty about the advantages of S-1[20-23]. Therefore, we attempted to assess the benefit of S-1-based chemotherapy through an exhaustive meta-analysis from all relevant trials.

MATERIALS AND METHODS

Aims

This meta-analysis systematically reviewed the published literature of randomized controlled trials, comparing the following therapies: S-1-based chemotherapy *vs* non-S-1-based chemotherapy; S-1-based chemotherapy *vs* 5-FU- or capecitabine-based chemotherapy in subgroup analyses.

Search strategy

S-1 and AGC were used as search terms. PubMed, the Cochrane library, Science Direct, EMBASE and American Society of Clinical Oncology meetings were retrieved, with a censor date up to November 2013. The search was limited to English language and human-based papers. Case-control and retrospective studies were excluded. To ensure that all relevant trials were included, we scanned related literature and references in the selected articles.

Study selection

We checked each article by viewing the title, abstract, and even the full text. Trials were included if they (1) were randomized controlled phase Ⅱ or phase Ⅲ trials; and (2) included patients receiving regimens which compared S-1-based regiments with non-S-1-based regiments given as first-line chemotherapy of AGC. We defined "advanced gastric cancer" as unresectable or recurrent or metastatic disease. Trials were excluded if patients also had radiotherapy, immunotherapy, or preoperative or intraperitoneal chemotherapy. Review articles, case reports, and letters were excluded. All different opinions were discussed. Complete articles of pertinent literature were used in this meta-analysis.

Data extraction

Author name, year of publication, chemotherapy regimens, objective response rate (ORR), prognosis and adverse events in eligible trials were extracted. The ORR was the percentage of patients who had a complete or partial tumor response. Time-related endpoints [overall survival (OS), progression-free survival (PFS) and time-to-treatment failure (TTF)] were used to measure prognosis. OS was defined as the time from random assignment to date of death from any cause. PFS was calculated from the date of randomization to the date of disease progression or death from any cause. TTF included progression, death or withdrawal. If necessary, we did a simple calculation to transform initial data into the forms suitable for meta-analysis. Likewise, data extraction was performed independently by two reviewers.

Statistical analysis

Time-to-event data (OS, PFS and TTF) were summarized using HR and 95%CIs. Dichotomous data (ORR and adverse events) were summarized using relative risks (RR) and 95% CIs. Stata software (version 12.0; Stata Corp LP, College Station, TX, United States) was used to calculate the pooled values.

Heterogeneity between studies was tested using χ^2 statistics and measured with the P value and I^2 statistic. I^2 lay between 0% and 100%, and a value of 0% indicated no observed heterogeneity, with larger values indicating increasing heterogeneity. The DerSimonian-Laird method (random-effects model) was used if heterogeneity existed and could not be explained or corrected. Otherwise, the Mantel-Haenszel method (fixed-effects model) was used. In the absence of heterogeneity, the fixed-effects and random-effects models provide similar results.

Forest plots were used to depict HRs and RRs within individual trials and overall. Begg's funnel plots were used to assess the potential publication bias by Egger's linear regression test. All P-values were two-sided at the 5% level, and CIs had two-sided probability coverage of 95%.

Table 1 Baseline characteristics

Study	Year	Country	Number of patients		Treatments	
			S-1	non-S-1	Experimental arm	Control arm
Ajani *et al*[24]	2013	Non-Asian	521	508	S-1: 25 mg/m^2, B.i.d, day 1-21; cisplatin: 75 mg/m^2, *civ* 1-3 h, day 1, q.4.w.	5-FU: 1000 mg/m^2/24 h, day 1-5; cisplatin: 100 mg/m^2, *civ* 1-3 h, q.4.w.
Huang *et al*[25]	2013	China	119	110	S-1: 80-120 mg/d, day 1-14; paclitaxel: 60 mg/m^2, *iv*, day 1, 8 and 15, q.4.w.	5-FU: 500 mg/m^2, *civ*, day 1-5; leucovorin 20 mg/m^2, *iv*, day 1-5; paclitaxel: 60 mg/m^2, *iv*, day 1, 8 and 15, q.4.w.
Kim *et al*[26]	2012	Korea	65	64	S-1: 80 mg/d, day 1-14; Oxaliplatin: 130 mg/m^2, *iv* (2 h), day 1, q.3.w.	Capecitabine: 2000 mg/d, day 1-14; Oxaliplatin: 130 mg/m^2, *iv* (2 h), day 1, q.3.w.
Nishikawa *et al*[27]	2012	Japan	80	77	(sequential), S-1: 80 mg/m^2, day 1-28, 2-wk rest followed by PTX; or (concurrent), S-1: 14 d and PTX: 50 mg/m^2, day 1, 8, q.3.w.	(sequential), intravenous 5-FU: 800 mg/m^2, *iv*, day 1-5, followed by weekly PTX at 80 mg/m^2; or (concurrent), 5-FU: 600 mg/m^2, *iv*, day 1-5 and weekly PTX at 80 mg/m^2, q.4.w.
Jeung *et al*[20]	2010	Korea	37	38	S-1: 35 mg/m^2, B.i.d, day 1-14; doc: 35 mg/m^2, day 1, 8, q.3.w.	cisplatin: 35 mg/m^2, day 1, 8; doc: 35 mg/m^2, day 1, 8, q.3.w.
Boku *et al*[22]	2009	Japan	234	232	S-1: 40 mg/m^2, B.i.d, day 1-28, q.6.w.	5-FU: 800 mg/m^2, *civ*, day 1-5, q.4.w.
Lee *et al*[23]	2008	Korea	45	46	S-1: 40 mg/m^2 (BSA < 1.25 m^2), 50 mg/m^2 (BSA: 1.25-1.5 m^2), 60 mg/m^2 (BSA > 1.5 m^2), B.i.d, day 1-28, q.6.w.	Capecitabine: 1250 mg/m^2, B.i.d, day 1-14, q.3.w.

5-FU: 5-fluorouracil; PTX: Paclitaxel; BSA: Body surface area.

RESULTS

Seven randomized controlled trials involving 2176 patients met the inclusion criteria and were included in this meta-analysis[20,22-27]. All the trials assessed adverse events according to the National Cancer Institute's common toxicity criteria. The details of the articles were summarized in Table 1.

S-1-based vs non-S-1-based chemotherapy

The HR summarizes survival for S-1-based compared with non-S-1-based chemotherapy, with an HR less than 1 indicating a survival advantage for S-1-based chemotherapy.

Compared to non-S-1-based regimens, the use of S-1-based regimens was associated with an increased ORR (RR = 1.300; 95%CI: 1.028-1.645). S-1-based chemotherapy had a marginal overall survival benefit compared to the control group (Figure 1), with a HR of 0.89 (95%CI: 0.81-0.99; P = 0.025). There was no significant heterogeneity between the studies (P = 0.263; I^2 = 22.7%). The PFS was not significantly better in the S-1-based group (HR = 0.84; 95%CI: 0.70-1.00; P = 0.052) (Figure 2), but TTF was significantly in favor of the S-1-based group (Figure 3), with a pooled HR of 0.83 from three related articles (95%CI: 0.75-0.92; P = 0.00). There was no significant inter-trial heterogeneity for the endpoints of TTF (P = 0.094; I^2 = 57.6%).

Six trials assessed adverse effects. Most grade 3 or 4 hematological and nonhematologic toxicities were not reduced in the S-1-based group. Only the risk of febrile neutropenia (RR = 0.225; 95%CI: 0.126-0.515; P = 0.00) and stomatitis (RR = 0.230; 95%CI: 0.060-0.878; P = 0.032) were lower with S-1-based chemotherapy than non-S-1-based chemotherapy. The details are listed in Table 2.

Only one of the trials, by Ajani *et al*[21], was from non-Asian countries. So we pooled the data from Asian countries, and found a longer OS (HR = 0.87; 95%CI: 0.75-0.99; P = 0.048), PFS (HR = 0.78; 95%CI: 0.68-0.89; P = 0.00) and TTF (HR = 0.76; 95%CI: 0.64-0.91; P = 0.003) in the S-1-based group. Only grade 3 or 4 leukopenia was less in the non-S-1-based chemotherapy (RR = 2.198; 95%CI: 1.403-3.443; P = 0.001).

S-1-based vs 5-FU-based or capecitabine-based chemotherapy

There were three standalone randomized controlled trials comparing S-1-based and 5-FU-based chemotherapy. Two trials assessed whether there were benefits of S-1-based *vs* capecitabine-based chemotherapy. In a subgroup analysis a pooled HR < 1 represents superiority of S-1-based chemotherapy. S-1-based chemotherapy increased ORR (RR = 1.454; 95%CI: 1.038-2.036; P = 0.029), and prolonged the OS and TTF compared with 5-FU-based chemotherapy, with HR of 0.895 and 0.832, respectively. However, no significant difference in PFS between the two groups was observed (HR = 0.809; P = 0.086). Also, S-1 had no advantage in ORR, OS, PFS and TTF over capecitabine (Table 3).

The incidence of leukopenia (RR = 0.584; P = 0.002) and stomatitis (RR = 0.230; P = 0.032) appeared to be higher in the 5-FU-based arm. The other grade 3 or 4 hematological and nonhematologic toxicities were not less in the S-1-based group. The frequency of these grade 3 or 4 adverse events did not differ between S-1-based and capecitabine-based chemotherapy. The details are listed in Table 4.

Publication bias

Begg's funnel plot and Egger's test were performed to assess publication bias. Studies were plotted in order of

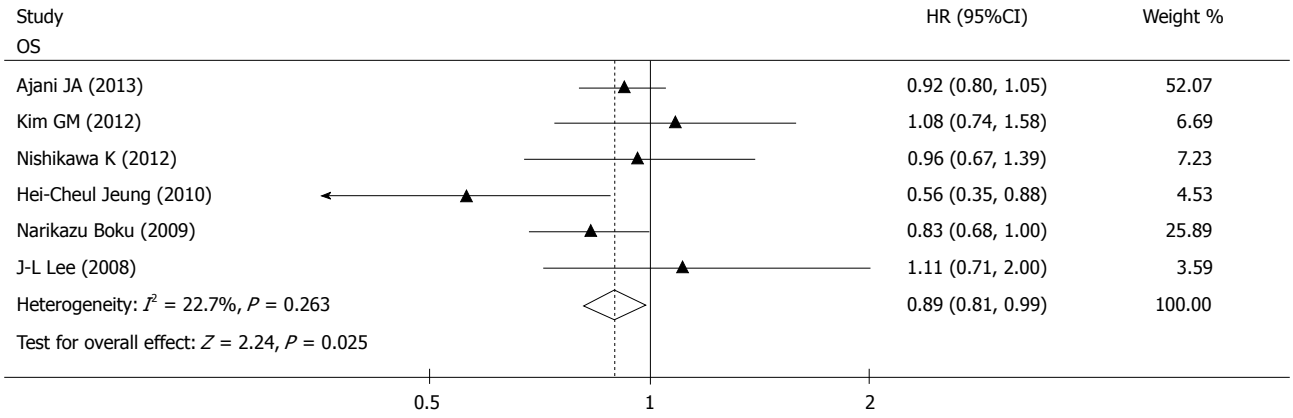

Figure 1 Comparison of overall survival between S-1-based chemotherapy and non-S-1-based chemotherapy. Values less than 1 indicate a survival advantage for S-1-based chemotherapy. OS: Overall survival; HR: Hazard ratio.

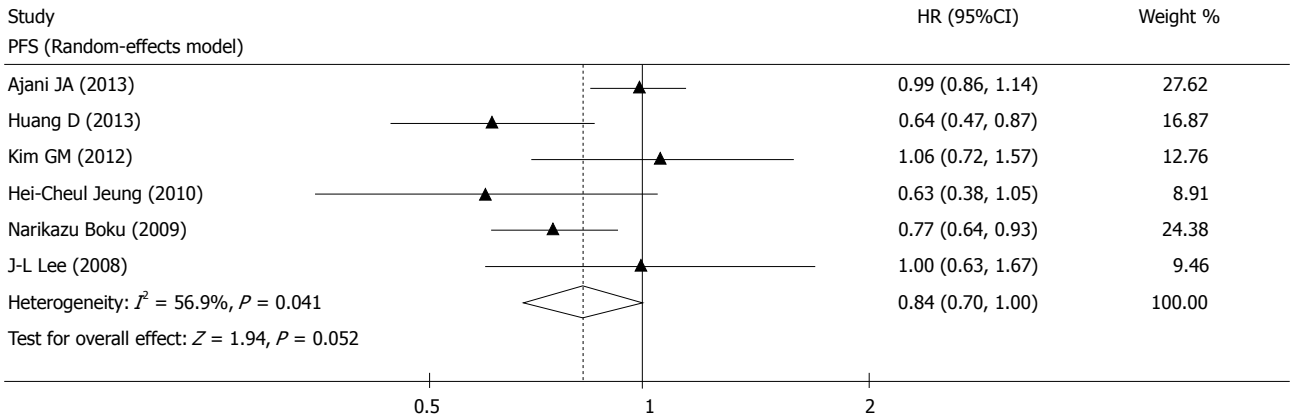

Figure 2 Comparison of progression-free survival between S-1-based chemotherapy and non-S-1-based chemotherapy. Values less than 1 indicate a survival advantage for S-1 based chemotherapy. PFS: Progression-free survival; HR: Hazard ratio.

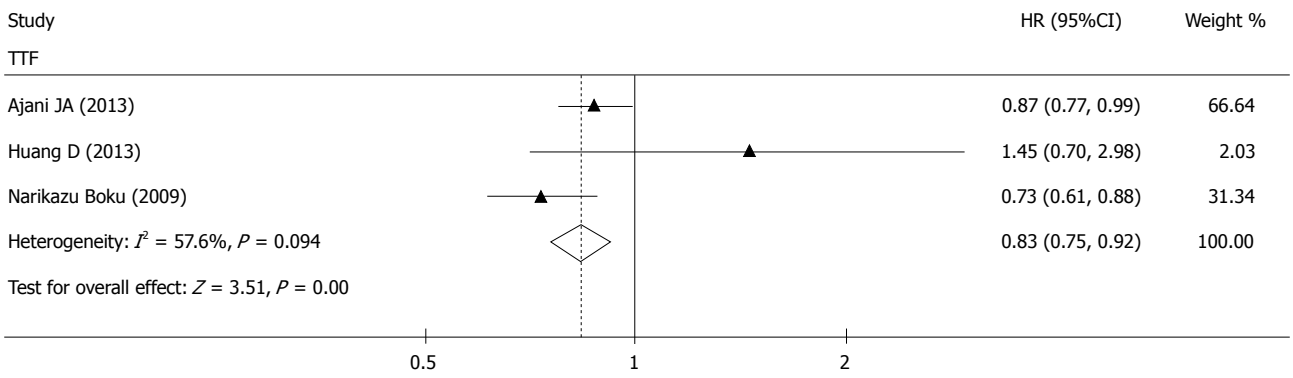

Figure 3 Comparison of time-to-treatment failure between S-1-based chemotherapy and non-S-1-based chemotherapy. Values less than 1 indicate a survival advantage for S-1-based chemotherapy. TTF: Time-to-treatment failure; HR: Hazard ratio.

decreasing variance of log HR. No publication bias was detected for all comparisons. Begg's funnel plots for the comparison of OS (Egger's test: P = 0.921; Begg's test: P = 0.851) are shown in Figure 4.

DISCUSSION

No standard chemotherapeutic regimens for AGC have been established worldwide as yet. Longer survival time, fewer adverse effects, better compliance and higher quality of life are sought. S-1, one kind of oral 5-FU, which offers convenience and tolerance for patients compared with traditional chemotherapy, may be an appropriate choice. Since S-1 was first approved by New Drug Application (NDA) in 1997 for chemotherapy of gastric cancer, numerous phase Ⅱ clinical trials and retrospective

Table 2 Comparison of toxicity between S-1-based chemotherapy and non-S-1-based chemotherapy

Toxicity	Number of Trials	Incidence of toxicity (%)		RR (95%CI)	*P* value
		S-1 Arm	Non-S-1 Arm		
Hematologic					
Anemia	6	14.01	14.86	1.150 (0.720-1.837)	0.560
Neutropenia	6	17.54	24.80	1.043 (0.451-2.413)	0.922
Thrombocytopenia	4	3.91	5.22	0.736 (0.499-1.085)	0.121
Leukopenia	6	8.87	9.15	1.334 (0.524-3.397)	0.546
Febrile neutropenia	3	0.86	3.54	0.225 (0.126-0.515)	0.000
Neutropenic infection	3	0.67	0.39	1.450 (0.476-4.424)	0.513
Nonhematologic					
Fatigue	6	8.67	8.37	1.041 (0.788-1.375)	0.777
Vomiting	5	4.29	5.61	0.769 (0.530-1.114)	0.164
Nausea	6	5.91	7.38	0.805 (0.583-1.111)	0.187
Diarrhea	6	5.24	3.54	1.288 (0.590-2.813)	0.525
Abdominal pain	2	4.00	2.76	1.469 (0.925-2.335)	0.103
Anorexia	6	7.44	6.99	1.074 (0.790-1.461)	0.647
Weight decreased	2	2.00	3.25	0.625 (0.369-1.061)	0.082
Stomatitis/mucosal inflammation	4	1.53	11.81	0.230 (0.060-0.878)	0.032
Liver function	3	0.86	0.69	1.221 (0.481-3.103)	0.674
Neuropathy, peripheral	5	0.67	0.89	0.724 (0.274-1.915)	0.515
Alopecia	2	0.38	0.30	1.205 (0.300-4.840)	0.792
Palmar-plantar erythrodysesthesia	4	0.38	0.59	0.719 (0.241-2.150)	0.555

RR: Relative risk.

Table 3 Comparison of objective response rate, overall survival, progression-free survival and time-to-treatment failure between S-1-based chemotherapy and 5-FU-based or capecitabine-based chemotherapy

Subgroups	ORR		OS		PFS		TTF	
	RR (95%CI)	*P* value	HR (95%CI)	*P* value	HR (95%CI)	*P* value	HR (95%CI)	*P* value
S-1 *vs* 5-FU	1.454 (1.038-2.036)	0.029	0.895 (0.805-0.995)	0.041	0.809 (0.635-1.030)	0.086	0.832 (0.751-0.992)	0
S-1 *vs* capecitabine	0.952 (0.649-1.397)	0.801	1.090 (0.803-1.481)	0.579	1.036 (0.764-1.405)	0.819	Not applicable	Not applicable

ORR: Objective response rate; OS: Overall survival; PFS: Progression-free survival; TTF: Time-to-treatment failure; 5-FU: 5-fluorouracil.

studies in Japan were started. An ORR of 20%-40%[28-31] and median OS of 250 to 350 d[30,32] were obtained for S-1 monotherapy in patients with AGC. The results were encouraging. So S-1 has been widely used in Japan for the treatment of AGC[33]. Recently, some phase Ⅱ and phase Ⅲ clinical randomized controlled trials both in Asian and non-Asian countries, compared S-1-based chemotherapy with non-S-1-based chemotherapy, and produced conflicting results. With limited sample sizes, it was difficult to draw definitive conclusions. A meta-analysis provides supreme evidence and a reliable answer to a clinical question, and this study pooled the data of 2176 patients from seven independent trials with a median follow-up about 2 years. This meta-analysis showed that S-1-based regimens were more effective than non-S-1-based regimens, with an absolute improvement of 11% in OS and 17% in TTF. The pooled HR also showed comparable PFS of the two treatments and slightly favored S-1-based therapy.

There are some limitations and explanations on the results. The impact of first line therapy on OS may be confounded by second-line or third-line therapies. However, follow-up treatments were not extensively reported in most of the eligible trials, so we could not analyze their possible impact on survival. However, follow-up treatments did not markedly alter TTF and PFS, which also confirmed the advantage of S-1-based chemotherapy. Another important factor influencing prognosis was follow-up time. By reviewing the included studies, we found most of the patients had passed away when follow-up ended and it indicated the follow-up was adequate. On the other hand, all the trials enrolled in this meta-analysis used daily administration of S-1, but it was demonstrated that, compared with daily administration, alternate-day administration of S-1 reduced adverse effects and provided sufficient clinical effects[34]. A retrospective study of alternate-day treatment with S-1 showed a response rate of 25%, with a median survival time of 338 d in patients with AGC[35]. In a mouse model, alternate-day treatment with S-1 was equivalent to daily treatment in terms of relative inhibition of tumor growth[36]. We hypothesize that alternate-day administration of S-1 may reduce adverse effects, improve compliance, and thus prolong survival time. Only one of the trials researched by Ajani *et al*[21] came from non-Asian countries. According to the suggestion of the reviewer, we pooled the data from Asian countries, and found longer OS, PFS and TTF for S-1-based treatment. Up to now, the only non-Asian global phase Ⅲ trial reported a negative result regarding survival time for S-1-based therapy. So the advantage of S-1 in the

Table 4 Comparison of toxicity between S-1-based chemotherapy and 5-fluorouracil- or capecitabine-based chemotherapy

Toxicity	S-1 *vs* 5-FU		S-1 *vs* capecitabine	
	RR	*P* value	RR	*P* value
Hematologic				
Anemia	1.073	0.794	1.914	0.145
Neutropenia	1.023	0.964	0.475	0.066
Thrombocytopenia	0.683	0.096	0.953	0.903
Leukopenia	0.584	0.002	1.788	0.402
Nonhematologic				
Fatigue	1.091	0.558	0.428	0.139
Vomiting	0.801	0.268	0.925	0.928
Nausea	0.791	0.179	1.177	0.812
Diarrhea	1.988	0.436	0.693	0.601
Anorexia	1.057	0.736	1.164	0.794
Weight decreased	0.625	0.082	Not applicable	Not applicable
Stomatitis/mucosal inflammation	0.230	0.032	Not applicable	Not applicable
Neuropathy, peripheral	0.808	0.722	Not applicable	Not applicable
Palmar-plantar erythrodysesthesia	1.770	0.468	0.193	0.133

5-FU: 5-fluorouracil.

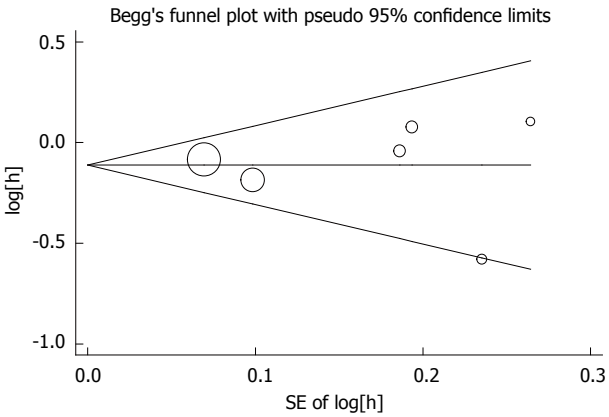

Figure 4 Begg's funnel plot of publication bias.

treatment of AGC is especially true in Asian population. The most relevant factor, in our opinion, is that the metabolic rate of conversion of S-1 to 5-FU seems to differ in various ethnic populations. S-1 is converted to 5-FU in the liver mainly by cytochrome P450 2A6 (CYP2A6). There are racial differences in CYP2A6 polymorphisms which affect the clinical outcomes of patients who are undergoing S-1-based chemotherapy for AGC[37]. Thus we think that the expression of specific genes may finally decide the effectiveness of S-1. For example, Ichikawa *et al*[38] found that treatment effects of S-1 monotherapy for gastric cancer are determined by the status of TS gene expression, regardless of DPD gene expression. Ishido *et al*[39] proved that intratumoral TS expression was an independent prognostic factor in patients with gastric cancer who received postoperative adjuvant chemotherapy with S-1. The predictive markers of S-1 should be further explored to guide rational clinical therapy.

We also paid close attention to the adverse effects. Most of the toxicities were predictable, tolerable and manageable, and only grade 3 or 4 adverse events were discussed. The use of S-1 did not increase the side effects and even reduced the rate of febrile neutropenia and stomatitis. As we known, S-1 improves the tumor selective toxicity of 5-FU especially by the actions of Oxo[40], an enzyme inhibitor of 5-FU, which can suppress the gastrointestinal toxicity of FT[12]. However, in this meta-analysis, we did not find a notable advantage of S-1 regarding gastrointestinal toxicities. The additional effect of concomitant chemotherapeutic agents, such as cisplatin and docetaxel may have affected the results.

Until now, 5-FU has comprised the backbone of chemotherapy for AGC. Oral fluoropyrimidines, such as S-1 and capecitabine, have opened new perspectives for the treatment of AGC with their simplicity and convenience over traditional 5-FU. So we evaluated their efficacy and safety to provide necessary and important information for clinical decision-making. Finally, S-1-based chemotherapy prolonged OS by 10% and TTF by 17% compared with 5-FU-based chemotherapy, and induced less leukopenia and stomatitis. We also found equivalent ORR, OS, PFS, TTF and grade 3 or 4 hematological and non-hematological toxicities in S-1-based and capecitabine-based chemotherapy. The new generation fluoropyrimidines, like S-1, may be a better choice than 5-FU in clinical use. Also, as they have similar antitumor efficacy and safety, we recommend that S-1 and capecitabine can be used for AGC interchangeably.

In our study, some limitations should be discussed. First, as with any meta-analysis, the study was not based on individual patient data and insufficient original data might limit the outcomes and cause confounding bias. We did our utmost to cover most reported endpoints in the randomized controlled trials and provide robust estimates. Second, heterogeneity between studies was present in this article, with a P-value < 0.05, especially in the evaluation of adverse effects. This was related to insufficient sample size and a shortage of some original data. We adjusted for this by using a trim-and-fill method in the random-effects model to make our outcomes statistically credible. Third, the numbers of published studies were not sufficiently large for a comprehensive analysis, particularly for the subgroup analysis, such as irinotecan- or paclitaxel-based regimens *vs* S-1-based regimens. Fourth, no trial showed the correlations between *H. pylori*-positive, Her2+, diffuse type or intestinal type, and the therapeutic effect of S-1, so we did not analyze these aspects in this article.

In conclusion, S-1-based chemotherapy may achieve the goal of longer survival and better tolerability than non-S-1-based chemotherapy as first line treatment for AGC. S-1 is an oral formulation and it is convenient for patients. We believe that S-1 plays an important role and may be a suitable choice in the therapy of AGC. More large scale randomized controlled trials need to be carried out to confirm the findings.

COMMENTS

Background

Gastric cancer remains the second leading cause of cancer-related death in the world. A standard chemotherapy regimen for advanced gastric cancer (AGC) is lacking. New-generation agents are being explored. S-1 is a novel oral formulation of 5-fluorouracil (5-FU). The efficacy and tolerability of S-1-based chemotherapy should be assessed.

Research frontiers

Based on the encouraging results from a number of phase Ⅱ trials for S-1-based chemotherapy, several phase Ⅱ and phase Ⅲ clinical randomized controlled trials, both in Asian and non-Asian countries, compared S-1-based-chemotherapy and non-S-1-based chemotherapy. However, there is controversy and uncertainty about the advantages of S-1.

Innovations and breakthroughs

This systematic review analyzed seven phase Ⅲ trials and 2176 AGC patients to compare S-1-based *vs* non-S-1-based chemotherapy and concluded that the use of S-1 was associated with an advantage in terms of objective response rate (ORR), overall survival (OS), time-to-treatment failure (TTF) and toxicities, especially in Asian populations. Similar results were found when comparing with 5-FU-based therapy. Furthermore, S-1-based regimens had no advantage in ORR, OS, progression-free survival, TTF, and adverse events over capecitabine-based regimens. The evidence might be used for future selection of S-1-based chemotherapy for AGC.

Applications

With longer survival, better tolerability, more convenient use for patients, S-1-based chemotherapy may be a suitable choice in the therapy of AGC.

Peer review

The manuscript provides a valuable meta-analysis result, offering suggestions for the S-1-based chemotherapy as a good choice for AGC. The work is well written and interesting because it focuses attention on a controversial issue in the treatment of AGC. Data selection and statistical method is considered as appropriate.

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CASE REPORT

Manifestations of gastrointestinal plasmablastic lymphoma: A case series with literature review

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Abstract

Plasmablastic lymphoma (PBL) rarely occurs in the gastrointestinal (GI) tract with limited studies reported. We reviewed the clinical histories and pathology of four patients with GI PBL at our institute and similar case reports published in peer-reviewed journals. In our first case, a 40 year-old human immunodeficiency virus positive male presented with a hemorrhoid-like sensation, and was diagnosed with PBL via biopsy of a rectal mass. The second case involves a 65 year-old healthy male with bloody diarrhea who was found to have PBL in a resected sigmoid mass. The third patient was a 41 year-old male with a history of Crohn's disease who presented with abdominal pain, diarrhea, and weight loss. A small intestinal mass (PBL) was removed. The fourth patient was a 65-year-old male who was found PBL after surgical resection of bowel for his florid Crohn's disease. He later developed secondary acute myeloid leukemia. Clinical outcome was very poor in 3 out of 4 patients as reported in the literature. One patient survived chemotherapy followed by autologous transplant. The prototypical clinical presentation and variations of PBL can help create a more comprehensive differential diagnosis for GI tumors and establish an appropriate therapeutic guideline.

Key words: Plasmablastic lymphoma; Undifferentiated carcinoma; Non-Hodgkin lymphoma; Diverse clinical manifestation and treatment

Core tip: Plasmablastic lymphoma rarely occurs as a primary lesion within the gastrointestinal tract. It frequently occurs in human immunodeficiency virus-positive patients, usually within the oral cavity. Its unique immunohistochemical profile may mislead unaware pathologists, and may potentially delay an accurate diagnosis and proper clinical treatment.

Luria L, Nguyen J, Zhou J, Jaglal M, Sokol L, Messina JL, Coppola D, Zhang L. Manifestations of gastrointestinal plasmablastic lymphoma: A case series with literature review. *World J Gastroenterol* 2014; 20(33): 11894-11903 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i33/11894.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i33.11894

INTRODUCTION

Plasmablastic lymphoma (PBL) is classified by the World Health Organization as a type of mature B-cell lymphoma that expresses plasma cell antigens (CD38, CD138, MUM1) but not common B-cell antigens (CD20, CD19, PAX5)[1,2]. While its pathogenesis is not yet fully understood it has been shown that the Epstein-Barr Virus (EBV) is present in a majority of cases and a small percentage of cases are associated with *MYC* gene rearrangement[2]. PBL was initially identified in the oral cavity of human immunodeficiency virus (HIV) positive individuals and this continues to be the prototypical presentation of approximately 80% of PBL cases within this population[3]. Since first described in 1997, there have been numerous cases in immunocompromised non-HIV individuals. PBL has also been found in areas outside the oral cavity, favoring sites such as the gastrointestinal (GI) tract, lymph nodes, and skin[4].

The gastrointestinal (GI) tract is one of the more common extranodal sites, especially in HIV negative patients. Given its unique phenotypic presentation by loss of common leukocyte antigen (CD45) expression, it might not directly lead to a diagnosis of conventional non-Hodgkin lymphoma, which is CD45 positive. Instead, initial differential diagnoses might include likely all CD45 negative neoplasms that could potentially involve the GI tract including poorly or undifferentiated carcinomas, including colorectal carcinoma, neuroendocrine cell neoplasms/carcinoid tumors, medullary carcinoma, signet ring cell carcinoma, metastatic tumors, angiosarcoma, *de novo* diffuse large B-cell lymphoma (DLBCL) and anaplastic plasmacytoma and rarely plasmablastic myeloma. Misinterpretation of GI PBL would have a great impact on treatment strategy and clinical outcome.

PBL is considered an aggressive lymphoma with a median overall survival of 14 mo[3,4]. There is no consensus on treatment protocol in place, and while more aggressive regimens are suggested, they have not shown to provide statistically significant improved outcome[5]. As of date, there are only a handful of case reports[6-19], but not a large case series emphasizing its clinical and pathologic variations as well as appropriate treatment implications.

CASE REPORT

Here we describe 4 cases of PBL found in the GI tract of patients who presented to Moffitt Cancer Center for evaluation and treatment. We have also conducted a review of the literature of other reported cases of GI plasmablastic lymphoma (GI-PBL).

Case 1

At an outside facility, a 40-year-old Caucasian male presented with complaints of recent onset weight loss, nausea, and a "hemorrhoidal type" sensation in his anal area. The patient was diagnosed with HIV in 2004 and attributed his initial weight loss to recent changes in his antiretroviral therapy (ART) therapy (lamivudine, tenofovir and efavirenz). He also had a history of hepatitis B virus infection. A mass was identified under endoscopy and was subsequently resected. A diagnosis of GI plasmablastic lymphoma (GI-PBL) was suspected by a surgical pathologist at the outside facility but the other neoplastic or non-neoplastic process involving GI tracts could not be completely excluded. The patient presented at our institution for evaluation one month after the resection for second opinion. The initial laboratory data showed a white blood cell count of 4.4 × 10^9/L, hemoglobin 133 g/L, and platelets of 145 × 10^9/L. His lactate dehydrogenase (LDH) level was slightly elevated at 952 U/L (normal range 313- 618 U/L). Pathologic review of the resected mass showed squamous mucosa covered tissue with a diffuse lymphoid infiltrate composed of intermediate-to-large cells with dispersed chromatin and prominent nucleoli, which showed round-to-slightly irregular nuclear borders (Figure 1A and B). Numerous single apoptotic bodies and tingible body macrophages were noted. Immunohistochemistry showed the atypical cells to be strongly positive for CD138 (Figure 1C), CD79a, BCL2, and BCL6. The tumor cells were also weakly positive for CD56 and CD45. *In-situ* hybridization was strongly positive for EBV-encoded RNA (EBER) (Figure 1D). The proliferation index was high (80%), as measured by a Ki-67 immunostain (Figure 1F). The specimen was negative for CD3, CD5, CD10, CD20 (Figure 1E), CD30, EMA, ALK, and human herpesvirus 8 (HHV-8). A fluorodeoxyglucose positron emission tomography (FDG-PET) scan showed hypermetabolic areas in the anal area, mediastinum, right hilum, retroperitoneum, and right common iliac chain. His bone marrow biopsy was unremarkable. Based on these clinical and pathologic findings, the final diagnosis was PBL. The patient was treated with dose-adjusted EPOCH (etoposide, vincristine, doxorubicin, cyclophosphamide, and prednisone) therapy, including intrathecal prophylaxis with methotrexate, which he tolerated very well. A follow-up computer axial tomography (CT) scan showed a decrease in his lymphadenopathy after 3 cycles. He was on ART and prophylactic antibiotics (Azithromycin, trimethoprim-sulfamethoxazole, and acyclovir). However, at his 12 mo follow-up, the patient had developed PBL of the bladder that was confirmed by biopsy. Per CT, massive nodal soft tissue involvements were noted within the left and right retroperitoneal regions of the pelvis anterior to the mid sacrum measuring (7.0 cm), periaortic region (2.0 cm) and adjacent to kidneys (6.0 cm). Additional chemotherapy was continued. However, the patient was lost of follow up after the visit.

Case 2

A 64-year-old male presented with bloody diarrhea. A pelvic CT with contrast was performed, which disclosed a large mass arising from the mid to distal sigmoid colon with prominent thickening of the sigmoid colon and exophytic extension (measuring 6.5 cm × 7.4 cm) into the soft tissues posterolateral to the sigmoid colon on the left side. An outside facility performed a colonoscopy and subsequent resection of the sigmoid mass. A diag-

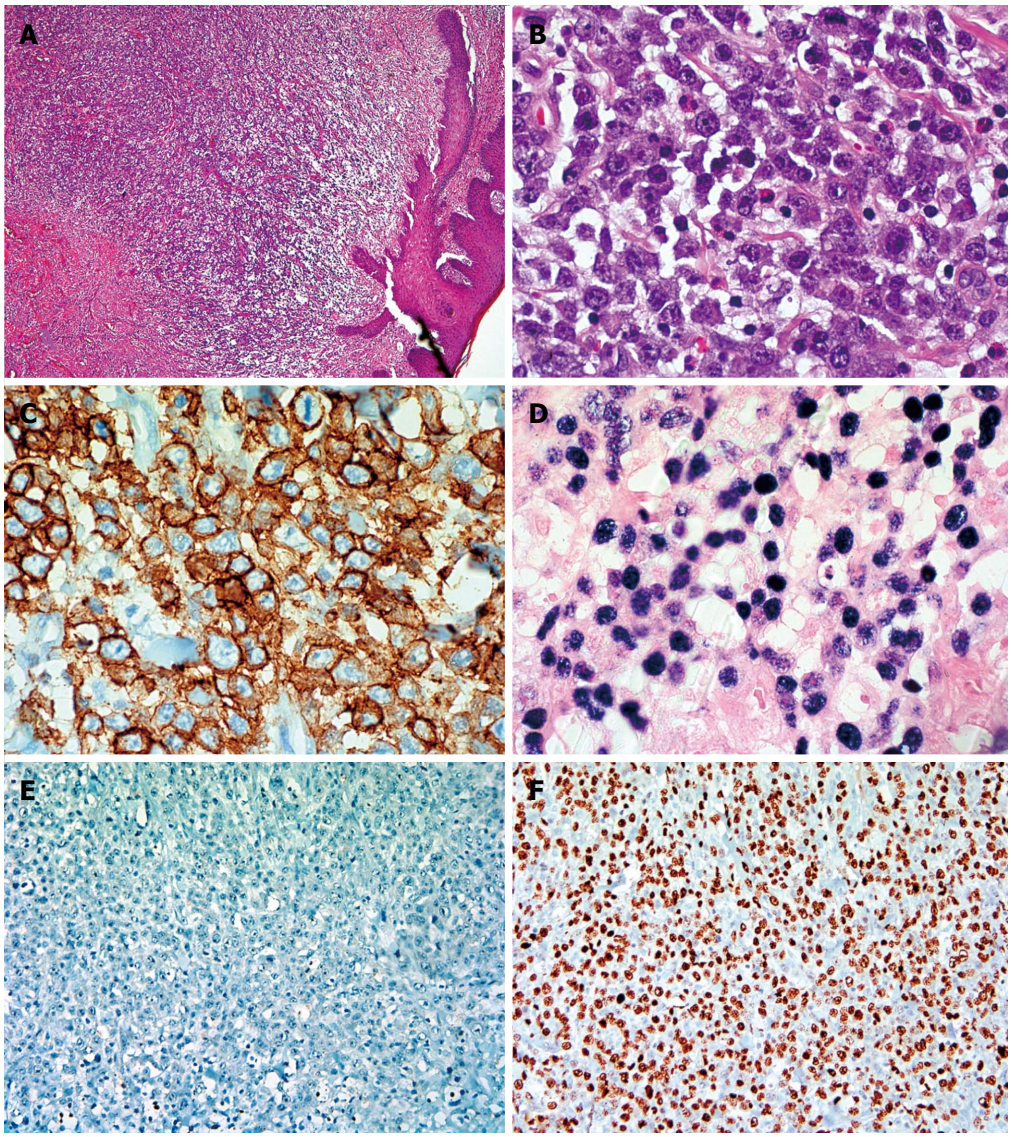

Figure 1 Microscopic examination. A: Microscopic examination of rectal biopsy showed intact squamous mucosa with submucosal dense lymphoid infiltrate associated with increased angiogenesis (HE, magnification × 40); B: High power view demonstrated sheets of large a typical lymphoid cells with plasmablastic differentiation (big round to oval nuclei, dense or disperse chromatin, prominent nucleoli and abundant amphophilic cytoplasm with increased apoptosis and mitosis and scattered inflammatory cells (HE, magnification × 600); C: CD138 immunostain highlighting the neoplastic cells (Immunoperoxidase, magnification × 600); D: *In situ* hybridization by using epstein barr virus -encoded RNA probe showed diffuse and strong signals (ISH, magnification × 600); E: Plasmablastic lymphoma cells being purely negative for CD20 (Immunoperoxidase, magnification × 100); F: High proliferation index was highlighted by Ki67 immunostain (approximately 80%) (Immunoperoxidase, Magnification × 100).

nosis of "favoring a hematopoietic tumor" was issued by the outside pathology facility. Differential diagnoses included, but was not limited to, anaplastic plasmacytoma, DLBCL and cytokeratin negative, poorly differentiated carcinoma. The patient was transferred to our hospital for consultation. Laboratory tests performed at our institution revealed a white blood cell count of 9.5 × 10^9/L, hemoglobin of 150 g/L, platelets of 309 × 10^9/L. His chemistry profile and liver function tests were normal. An HIV test was negative. The slides of bowel resection were reviewed and further ancillary studies were ordered. Microscopic examination of the resected specimen showed atypical plasmacytoid cells with intermediate to large nuclei with peripheral chromatin and many prominent nucleoli. The atypical cells were positive for CD45 (dim), CD10, CD38 (bright), and VS38. There was focal positivity for EMA, BCL6, CD30, and cyclin-D1. CD117 staining showed weak positivity. They were negative for CD19, CD20, CD5, CD79a, CD138, CD34, and HLA-DR. According to the outside pathology report, the neoplastic cells were negative for cytokeratin AE1/AE3, CK7, CK20, CAM5.2, CDX2, melan A, S100, ALK, BCL2, CD34, CD4, CD7, CD56, CD68, and TdT. Light chain studies were non-contributory due to heavy background staining. The proliferation fraction by Ki-67 was estimated to be greater than 90%. Additional studies performed at our institution confirmed that the tumor was negative for CD20 and strongly positive for EBER. While EBV associated DLBCL of the elderly can be CD20 negative and present with immunoblastic fea-

tures resembling PBL, it is usually non-germinal center type, and this patient also showed negativity for CD19, CD79a, and BCL2 and positivity for CD10 and BCL6. These findings made this a less likely diagnosis, and the final diagnosis was PBL. A FDG-PET scan showed hypermetabolic areas in the pelvis. A bone marrow biopsy showed normocellular bone marrow with no evidence of involvement by malignant lymphoma. He was initially treated with R-CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone-rituximab) therapy because the immunohistochemistry (IHC) panel received from the referring entity did not contain a CD20 stain. The clinician thought it would be prudent to add rituximab to the patient's therapy until our institution ran an additional IHC study to confirm that the lymphoma cells completely lacked CD20 expression. The patient went into complete remission after six cycles of chemotherapy and received an autologous hematopoietic stem cell transplant (auto-HSCT). Follow-up 44 mo after the initial diagnosis and 35 mo post transplant showed complete remission.

Case 3

A 41-year-old male with a longstanding history of Crohn's disease diagnosed in 1999 had undergone a right colectomy almost 10 years prior to presentation. He was on 6-mercaptopurine (6-MP) and budesonide (Entocort) since 2001, and was clinically stable under his primary care physician besides a chronic fistula-in-ano for the last two years. He presented with a 35 pound weight loss, abdominal pain, and diarrhea at a local hospital, where a CT of the abdomen demonstrated diffuse bowel wall thickening of small bowel loop and associated with a mass showing irregular central area of non-enhancement (phlegmon) measuring 7.0 cm × 4.0 cm around the small intestine. The patient was initially treated with steroids and antibiotics without improvement. The involved section of bowel was removed after a CT-guided needle biopsy failed to drain any fluid from the mass. The patient came to our institution for further evaluation and treatment. A microscopic review of the mass showed mucosa-covered tissue associated with focal dense infiltrates of sheets of large atypical lymphoid cells throughout the whole thickness of the small bowel wall, which was superimposed with inflammatory process. The atypical cells had eccentrically round to oval nuclei, prominent nucleoli, and abundant eosinophilic cytoplasm. The tumor cells stained positive for CD30, CD79a, MUM-1 and weakly positive for PAX5 and CD68. There was variable staining for CD45, CD20, CD138, CD3, CD5, CD25, ALK1, cytokeratin, CAM5.2, and CD56 were negative. EBER was positive. Ki-67 showed a proliferation index of 75%. This confirmed the diagnosis of a primary GI PBL. The uninvolved segments of small bowel display characteristic histology for Crohn's disease. Bone marrow staging was negative for involvement by lymphoma. A FDG-PET/CT scan demonstrated increased metabolic activity within loops of proximal large intestine and no evidence of metabolically active lymphadenopathy within the neck, chest, abdomen or pelvis. The patient was started on HyperCVAD chemotherapy (cyclophosphamide, vincristine, doxorubicin and dexamethasone). Before starting cycle 3 of the chemotherapy regimen, he was found to have a mass on his bladder wall. After 3 cycles of chemotherapy, a CT scan showed growth of the bladder mass with biopsy confirming PBL. He was started on radiation therapy but unable to continue due to declining health. The patient was discharged to hospice care and subsequently died of disease.

Case 4

A 65-year-old white male presented to our clinic in 2011 with a history of Hashimoto's thyroiditis diagnosed in 1988, status post right thyroidectomy in 1999, and a history of Crohn's disease (CD) diagnosed in 2000, which was treated with oral steroids, infliximab and budesonide. He also underwent left thyroidectomy for a thyroid nodule in January 2006, which revealed a limited-stage of low-grade extranodal marginal zone lymphoma (MZL) requiring no additional treatment. In June 2011, he developed acute small bowel obstruction, which was initially considered to be associated with exacerbation of Crohn's disease. He underwent resection of the ileum and cecum. Unexpectedly, the histology and immunohistochemistry studies reported a multifocal involvement of ileum and cecum with PBL [CD138 (+), partial CD79a (+), focally CD20 (+), kappa light chain (+), lambda light chain (-) and high proliferation index, 100%, highlighted by Ki67] in addition to Crohn's disease. All 14 biopsied lymph nodes were free of lymphoma. Staging bone marrow biopsy showed no involvement with lymphoma. ELISA testing for HIV-1 and 2 was negative. A CT scan of the abdomen revealed a new soft tissue mass along the right kidney. He was treated with two cycles (4 arms) of hyperCVAD and rituximab between August and October 2011 and achieved complete remission, per restaging reports. He was consolidated with conditioning regimen BEAM+R (BCNU, etoposide, ara-C and melphalan and rituximab), followed by auto-HSCT in November 2011. His post-transplant course was complicated with protracted moderate to severe thrombocytopenia ranging from 3.0-53.0 × 10^9/L that required platelet transfusions. He had mild normocytic anemia and normal absolute neutrophil count. The patient was treated with steroids, danazol and thrombopoietin receptor agonist (Eltrombopag) for a possible immune-mediated peripheral destruction of platelets given sustained thrombocytopenia and normal bone marrow findings (2 biopsies within 6 mo) but did not achieve a durable response. Subsequent restaging FDG-PET/CT scan performed in February 2013 showed no evidence of recurrent PBL. However, a repeat bone marrow biopsy in May 2013 revealed hypercellularity (60%) with increased myeloblasts (40%) and cytogenetic abnormalities including del (7q) and isochromosome 9 in 2/30 cells. Fluorescent *in situ* hybridization (FISH) also demonstrated both del(7q31) and del(5q31). The overall findings supported a diag-

nosis of therapy-related acute myelogeneous leukemia (tAML). He underwent induction chemotherapy with CLAG (cladribine, ara-C and G-CSF), but his day 14 bone marrow biopsy revealed residual leukemia. His hospital course was complicated with bacterial and fungal infections along with significant physical deconditioning. Although the patient was offered reinduction therapy, he opted for comfortable measures with hospice. He succumbed to tAML two months later. A comparison of the clinical and pathological features in our four cases is summarized in Table 1.

Literature review of gastrointestinal plasmablastic lymphoma

A total of 14 cases of GI PBL were found during our search of PubMed. The publication dates range from 1998[10] to 2013[13]. The clinical and pathological characteristics of the published cases are summarized in Table 1. The median age of the reported cases is 57 (ranging 17 to 82) with a male-to-female ratio of 3:4. The most common symptoms at presentation were abdominal pain (57%), weight loss (50%), anorexia (36%), and melena (36%). The other B symptoms of fever and night sweats were present in 29% and 7% respectively. Overall, 71% of the patients displayed B symptoms. However, only one patient displayed all three[6], and this patient was HIV-positive. Other symptoms included abdominal distention, diarrhea, vomiting, and rectal bleeding. The locations of these primary lesions consisted of the stomach (43%), small intestine (21%), anal region (21%), cecum (14%), colon (7%), and esophagus (7%). Of the fourteen cases, 4 were HIV-positive, 9 were HIV-negative, and 1 patient's status was unknown. The immunophenotype, EBV/HHV-8 infection, and c-MYC status for these cases are presented in Table 2. Only 8 of the cases were tested for the presence of EBV. Of these cases, 4 (50%) were positive. Three of these four EBV-positive cases were also HIV positive. The fourth had an unknown HIV status. HHV8 was only tested in HIV positive patients, with 75% of them to be reportedly positive. The most common therapy administered was CHOP (57%). Other chemotherapy regimens included LACE (cyclophosphamide, cytarabine, etoposide, and lomustine), EPOCH, and ProMACE-cytaBOM (cyclophosphamide, doxorubicin, etoposide cytozar, bleomycin, vincristine, methotrexate, and prednisone). Of the cases where no chemotherapy was mentioned (Nos 7, 11 and 14), one patient died before therapy could be started, one case was treated with high dose steroids only and died within 2 wk of presentation, and the last case did not have a record of treatment. The median survival (in months) for ten of the patients with available data was 3.25 mo. More than 50% (6 of 11) died of disease shortly after diagnosis or post chemotherapy (Table 1). There was only one patient alive on a 5-year follow-up.

DISCUSSION

Plasmablastic lymphoma tends to present in the oral cavity of HIV-positive patients. In HIV-negative patients, extranodal, mucosal areas are the most common primary sites[4]. A diagnosis of PBL of GI tract could be difficult since morphologically PBL could mimic a poorly differentiated carcinoma, DLBCL, Burkitt lymphoma, plasmacytoma and EBV associated DLBCL of the elderly. The morphologic spectrum of PBL includes immunoblastic, Burkitt, and plasmacytic variants. Of note, there are morphologic variations among the current cases. In case 1, the tumor cells are Burkitt-like, intermediate to large in size with dispersed chromatin, brisk mitoses, and increased apoptosis. The lymphoma cells in cases 2, 3 and 4 are predominantly immunoblastic, centroblastic or focally mixed with the two components. Phenotypically, some PBL lacks or expresses weak CD45, which could be misinterpreted as carcinoma if extensive ancillary studies are not performed; a small subset of PBL retains CD20 expression also results in diagnostic difficulty to separate it from *de novo* DLBCL, not otherwise specified (NOS) when encountering a limited biopsy sample. Thus, comprehensive immunophenotyping is necessary to distinguish PBL from the other neoplasms[20,21].

PBL could also be linked to immunocompromised status including the elderly. Both immunocompromised and immunocompetent patients tend to present with late stage disease[2]. B symptoms are symptoms associated with lymphoma and include weight loss, fever, and night sweats. These symptoms are more commonly reported in HIV-negative (50%) patients as compared to HIV-positive patients (33%)[4]. While not all patients with GI PBL presented with B symptoms, they all had gastrointestinal symptoms or signs (Table 1). Without imaging studies and biopsy, many could be missed at an earlier course in the disease. Moreover, of the 4 cases of primary GI PBL that presented to our institution and the 14 reported cases in the literature, 39.5% of the patients (5 of 13, 1 not available) had a known HIV-positive status. For the other case studies, it is unclear if any of the patients were immunosuppressed due to other reasons that could be attributed to disease development or progression. Three of the 14 patients were previously diagnosed with other neoplasms, including adenocarcinoma of the colon[15], meningioma[14], and squamous cell carcinoma of the maxillary sinus[8]. However, there were no details with regard to chemotherapy treatment, radiation or immunomodulation. Cases 3 and 4 from our institution had a history of Crohn's disease that developed PBL. To our knowledge, this is the first report of PBL arising in the GI tract of 2 patients with inflammatory bowel disease. The incidence of non-Hodgkin lymphoma within the inflammatory bowel disease population is common in the literature. There is debate as to whether the increased risk of non-Hodgkin lymphoma in these patients is related to their disease, use of immunomodulators, or the use of anti-Tumor Necrosis Factor (anti-TNF) medications. Biancone *et al*[22] found in their cohort study that immunomodulators and anti-TNFs did not increase overall cancer risk for patients with Crohn's disease, but that a

Table 1 Literature review and case study summary of demographic, clinical presentation, treatment and outcomes of plasmablastic lymphoma of gastrointestinal tract

No.	Study	Year	Age/ sex	Tumor location	HIV status	GI S/Sx	B symptoms	Chemotherapeutic treatment	Response	Outcome
1	Mani *et al*[6]	2008	40/M	Esophagus, stomach	+	Progressive odynophagia	WL, F, NS	LACE	Remission	Alive at 6 mo
2	Cha *et al*[7]	2010	60/M	Small intestine	-	Dyspnea, melena	WL +	CHOP	PR	Relapse, alive at 24 mo
3	Brahmania *et al*[18]	2011	59/M	Ano-rectal	-	Painless rectal bleeding	NR	CHOP	Remission	Alive at 5 yr
4	Mihaljevic *et al*[17]	2012	60/M	Stomach	-	Melena[1]	WL +	CHOP	UR	DOD after one cycle of CHOP
5	Hashimoto *et al*[15]	2012	70/F	Stomach	-	Melena	NR	CHOP, VP 16, ifosfamide, carboplatin	UR	Died during treatment
6	Chapman-Fredricks *et al*[14]	2012	46/F	Stomach	+	N/V, diarrhea, melena	NR	EPOCH	NL	NL
7	Bahari *et al*[11]	2012	17/F	Small intestine	ND	Diarrhea, distention[1]	F+	NL	NL	DOD before diagnosis made
8	Pruneri *et al*[10]	1998	53/F	Stomach	-	Postprandial fullness, stomach ache	F+	ProMACE-cytaBOM	Remission	Alive at 19 mo
9	Rajagopal *et al*[9]	2006	35/M	Ano-rectal	+	Rectal bleeding, tenesmus, constipation[1]	WL +	CHOP	Remission	Alive at 5 mo
10	Wang *et al*[8]	2012	55/M	Small intestine	-	Distention, vomiting, anorexia[1]	WL +	CHOP	UR	DOD at 1.5 mo
11	Hatanaka *et al*[19]	2010	75/M	Cecum	-	NL[1]	F +	NL	NL	NL
12	Lim *et al*[16]	2009	47/F	Ano-rectal	+	Anal bleeding, pain, fistula for 1 yr	NR	CHOP	Received 3 cycles of chemotherapy, status unknown	NL
13	Marques *et al*[13]	2013	82/F	Stomach	-	Melena, epigastric pain, abdominal fullness	WL +	CHOP	UR	DOD after 1 cycle of chemotherapy
14	Mansoor *et al*[12]	2012	77/F	Cecum, colon	-	Recal bleeding, diarrhea, vomiting[1]	WL +	NL	UR	DOD 3 wk after presentation
Case 1	Luria *et al*	Current case	40/M	Rectal	+	Hemorr-hoids like sensations	WL +	EPOCH	PR	Relapsed PBL in pelvic, abdomen, bladder 1 yr after PBL diagnosis - loss of follow after it
Case 2	Luria *et al*	Current case	64/M	Sigmoid colon	NR	Bloody diarrhea	NR	CHOP-R, Auto-HSCT	CR	Alive at 44/35 mo
Case 3	Luria *et al*	Current case	41/M	Terminal ileum	NR	Abdominal pain, diarrhea, 35-pound weight loss	WL +	Hyper-CVAD + Velcade	UR	Progressive, bladder mass, hepatic metastases, DOD, 17 mo after PBL diagnosed
Case 4	Luria *et al*	Current case	65/M	Terminal ileum and cecum	-	Acute bowel obstruction	NA	Hyper-CVAD + rituximab, auto-HSCT	UR	CR for PBL, but developed tAML and DOD 2 mo after tAML diagnosed and 25 mo after PBL diagnosed

[1]Accompany abdominal pain; 44/35: 44 mo after diagnosis and 35 mo post transplant; +: Positive; -: Negative; ND: Not done; GI S/Sx: GI tract symptoms and signs; WL: Weight loss; F: Fever; NS: Night sweats; NR: None reported; NL: None listed; CHOP: Cyclophosphamide, hydroxydaunorubicin, oncovin, prednisone; CHOP-R: Cyclophosphamide, doxorubicin, vincristine, prednisone-rituximab; LACE: Lomustine, cytarabine, cyclophosphamide, etoposide; EPOCH: Etoposide, vincristine, doxorubicin, cyclophosphamide, and prednisone; proMACE-cytaBOM: Cyclophosphamide, doxorubicin, etoposide cytozar, bleomycin, vincristine, methotrexate and prednisone; Hyper-CVAD: Fractionated cyclophosphamide, vincristine, doxorubicin and dexamethasone; PR: Partial remission; UR: Unresponsive to therapy; NA: Not Applicable; DOD: Died of disease.

fistulizing pattern of disease did. A recent case report also addressed a concern about whether using infliximab (anti-TNF) for inflammatory bowel disease (ulcerative colitis) was associated with a significantly increased risk of developing lymphomas[23]. However, a meta-analysis by Siegel *et al*[24] did not show a statistically increased risk of non-Hodgkin lymphoma in patients who were treated with anti-TNF and immunomodulator as compared to the general population. They were unable to establish a clear-cut link and expressed concern that age might be a confounding variable. Our patient had a long history of treatment with steroids (*Budesonide*) and/or 6-mercaptopurine. Both drugs are immunosuppressive[25,26]. Immunosuppressed status, in conjunction with reactivation of EBV, could be inciting factors for PBL with adverse clinical outcomes.

The presence of EBV in PBL has been cited repeatedly, and there is interest if this represents a route of pathogenesis. EBV has been shown to be involved in malignant transformation in a number of other lymphoproliferative disorders including Burkitt lymphoma and Hodgkin lymphoma[27]. It appears the viral-encoded product LMP1 has been linked to growth and proliferation[28]. This membrane bound protein is functionally similar to CD40 and is constitutively active. Moreover, since DNA methylation occurs ubiquitously in human cancer from the earliest measurable stages, a novel study of Hansen KD group revealed that extensive blockage of hypomethylation occurred in EBV-induced B-cells, which could be another reason for their immortalization[29]. EBV RNA is present in the majority of HIV-positive PBL cases and about half of HIV-negative cases[4]. Right now, the presence or absence of EBER is being used to help diagnose PBL. However, there have been recent reports of clinicians attempting to track their patient's response to treatment and possible relapse by measuring EBV DNA viral load in blood samples at different intervals[30,31]. They found that lower levels from diagnostic baseline corresponded with remission, while higher levels corresponded with relapse.

Researchers have also sought to understand the pathogenesis of PBL by understanding its common genetic anomalies. The proto-oncogene *c-MYC* is frequently observed in a variety of tumors. *C-MYC* is a well-studied phosphoprotein whose rearrangement has been associated with poor clinical outcome[32]. It is most commonly associated with Burkitt lymphoma but is seen in other malignancies as well[33]. In a study conducted by Valera *et al*[34], 49% of the 41 cases of PBL that were able to be investigated demonstrated rearrangement of *c-MYC*. Five of these patients had PBL of the GI tract, and of those, two showed rearrangements with immunoglobulin heavy chain (*IgH*) and one showed gains in *c-MYC* expression. The most common rearrangement encountered in the literature is between *c-MYC* and *IgH*[32-34]. *c-MYC* status was not well investigated in PBL. In an article describing 3 cases of PBL with *c-MYC*/*IgH* rearrangement by Bogusz *et al*[33], they found that these patients had extremely low CD4 counts (21, 48, and 35 cells/mm^3) as compared to 6 PBL patients without the rearrangement. In these 6 patients, the median CD4 count was 300 cells/mm^3. One of the 3 *c-MYC*/*IgH* rearrangement cases PBL was found in the anus and bone marrow. The case was not included in our review set because it was part of a table, and a full patient history was not present. Of 14 listed cases (Table 2) and all 4 cases at our institution, only one study was analyzed by fluorescence *in-situ* hybridization (FISH) for *c-MYC*, which was positive. Since a dysregulated *c-MYC* gene could be a potential therapeutic target[35,36], further investigation of *c-MYC* in PBL may potentially have important therapeutic and prognostic implications.

One of the reasons patients with PBL may not present with B symptoms can be explained by a proposed mechanism for its pathogenesis. B lymphocyte-induced maturation protein-1 (BLIMP1) and X-box-binding protein 1 (XBP1) are proteins that serve as reliable plasma cell markers because they are involved in terminal B-cell differentiation[4,37]. They have come to be recognized as markers of PBL because the morphology and immunoprofile of this disease can overlap with other entities such as multiple myeloma, DLBCL, and primary effusion lymphoma[1,4,38]. As BLIMP1 expression increases there has been shown to be a correlative decrease in expression of human leukocyte antigen DR (HLA-DR) and by association major histocompatibility complex class Ⅱ (MHC Ⅱ)[37,38]. MHC Ⅱ is involved in recruiting and activating tumor-infiltrating T cells which could potentially help combat tumor growth and contribute to the manifestation of B symptoms. Loss of this protein has been linked with more aggressive forms of DLBCL[37] and it has been theorized that this may contribute to PBL's behavior.

Chemotherapy regimens that have been used with partial or complete response include CHOP, infusional EPOCH, hyperCVAD, and CODOX-M/IVAC[4]. Antiviral therapy and monitoring of HIV viral titers and CD4 count also play a critical role in the treatment of HIV positive patients. Aggressive chemotherapy regimens have not shown to produce a statistically significant improvement in outcome[5]. Further study of the occurrence of MYC dysregulation in PBL may help us better understand the disease mechanism and guide future pharmacological research and chemotherapeutic regimens. Until a more focused treatment option can be provided to patients, there has been some success with adding bortezomib to the current regimens or having the patients undergo auto-HSCT should they achieve complete remission on therapy[5,39]. Case 2 in our study was treated with auto-HSCT after his chemotherapy and showed the longest remission of the 3 (44 mo). Case 4 achieved complete response for his PBL but developed secondary AML two years after auto-HSCT. To date, no study has specifically analyzed the overall survival of auto-HSCT for the treatment of PBL. Liu *et al*[39] reported four patients who received auto-HSCT after chemotherapy at their institution and showed a median survival of 27.5 mo.

Table 2 Literature review and case study summary of immunophenotypic variation and available Epstein-Barr Virus/HHV8 data of plasmablastic lymphoma of gastrointestinal tract

No.	Study	CD45	CD20	CD79a	PAX5	CD38	CD138	MUM-1	Ki-67	EBER	MYC	HHV8
1	Mani *et al*[6]	+	-	+ (W)	-	ND	+	ND	ND	+	ND	-[1]
2	Cha *et al*[7]	ND	-	+ (F)	ND	ND	-	+	70%	-	ND	ND
3	Brahmania *et al*[18]	-	-	-	ND	+	+	+	70%	+	ND	ND
4	Mihaljevic *et al*[17]	ND	-	ND	ND	-	-	+	70%	-	ND	ND
5	Hashimoto *et al*[15]	-	-	-	-	ND	+	+	100%	-	ND	ND
6	Chapman-Fredricks *et al*[14]	-	-	-	-	ND	+	+	> 90%	+	+	-[1]
7	Bahari *et al*[11]	+	-	+	ND	ND	+	ND	ND	ND	ND	ND
8	Pruneri *et al*[10]	-	-	-	ND	+	ND	ND	50%	ND	ND	ND
9	Rajagopal *et al*[9]	+	-	-	ND	+	ND	ND	80%	ND	ND	ND[1]
10	Wang *et al*[8]	-	-	+	ND	+ (F)	+	-	80%	-	ND	ND
11	Hatanaka *et al*[19]	-	-	-	ND	ND	+	ND	90%	ND	ND	ND
12	Lim *et al*[16]	ND	-	ND	ND	ND	+	ND	95%	+	ND	+[1]
13	Marques *et al*[13]	-	-	ND	ND	ND	+	+	90%	ND	ND	ND
14	Mansoor *et al*[12]	+ (W)	-	+ (W)	ND	ND	+	ND	90%	ND	ND	ND
Case 1	Luria *et al*	+ (W)	-	+ (W)	ND	ND	+	ND	80%	+	ND	-
Case 2	Luria *et al*	+ (W)	-	-	ND	+	-	ND	> 90%	+	ND	ND
Case 3	Luria *et al*	-/+	-	+	+	ND	+	+	75%	+	ND	ND
Case 4	Luria *et al*	ND	-	+	-	ND	+	ND	100%	ND	ND	ND

[1]HIV+ patients (x 4). +: Positive; -: Negative; ND: Not done; (W): Weak; (F): Focal; NA: Not applicable; EBER: Epstein-Barr Virus -encoded RNA.

In summary, clinical manifestations and treatments are varied among cases. Understanding the rare disease entity would benefit patient care by rendering earlier correct diagnosis, predicting clinical outcome and taking appropriate therapeutic strategies. A large scale study with standard approaches is eventually needed.

COMMENTS

Case characteristics

Plasmablastic lymphoma of gastrointestinal tract (GI-PBL) is a rare variant of B-cell non-Hodgkin lymphoma with an aggressive clinical course and shorter overall survival of 1-2 years, which is primarily associated with human immunodeficiency virus (HIV) infection but can also be seen in immunecompromised status including the elderly.

Clinical diagnosis

It often presents with gastrointestinal (GI) symptoms and signs such as bloody stool, diarrhea, abdominal pain accompanied with or without weight loss.

Differential diagnosis

Differential diagnoses should include, but not be limited to, poorly differentiated carcinomas, of GI tract, metastatic neoplasms, some sarcomas, *de novo* diffuse large B-cell lymphoma, and plasma cell neoplasms.

Laboratory diagnosis

Laboratory investigations should include routine CBC, serum lactate dehydrogenase level, and virology including HIV and Epstein-Barr Virus.

Imaging diagnosis

Imaging study using positron emission tomography scan/computer axial tomography scan revealed a mass with or without bowel obstruction.

Pathological diagnosis

PBL poses a diagnostic challenge given its unique immunophenotypic profile (negative for CD45, B-cell markers, CD20, PAX-5, and positive for plasma cell markers such as CD138, CD38, MUM1 and partially CD79a) and a high proliferation index. Thus, a comprehensive study including careful morphologic examination, extensive immunophenotyping (immunohistochemistry or flow cytometry) and cytogenetic/FISH study should be completed before the diagnosis is rendered.

Treatment

Although both CHOP and EPOCH are considered the common therapeutic choices, standard therapy or treatment guidelines have not been established yet. Autologous transplant is considered optional and tends to have a good outcome but still limited in experience.

Term explanation

Anaplastic plasmacytoma: a morphologic variant of plasmacytoma. Plasmablastic myeloma: a morphologic variant of plasma cell myeloma/multiple myeloma, often involving bone marrow, bone, soft tissue and rarely found in GI tracts as extramedullary presentation.

Experiences and lessons

Given GI-PBL being a great mimicker for the other GI neoplasms, lessons we learned are to include rare entity into initial differential diagnoses, in particular for the patients who are in immunosuppressant(s) or immunocompromised status.

Peer review

This article is writen very well as pathological case report. The authors have presented four cases of primary GI large B-cell lymphomas with immunophenotype and Epstein-Barr Virus expression consistent with plasmablastic lymphoma. Only one of the four cases were HIV positive and a second patient had iatrogenic immunosuppression. They have also summarized clinicopathological findings of 14 cases of primary GI plasmablastic lymphoma review of literature. They discuss the possible molecular and immune mechanisms that may lead to this uncommon tumor.

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CASE REPORT

Total pancreatectomy for metachronous mixed acinar-ductal carcinoma in a remnant pancreas

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Abstract

In October 2009, a 71-year-old female was diagnosed with a cystic tumor in the tail of the pancreas with an irregular dilatation of the main pancreatic duct in the body and tail of the pancreas. A distal pancreatectomy with splenectomy, and partial resection of the duodenum, jejunum and transverse colon was performed. In March 2011, a follow-up computed tomography scan showed a low density mass at the head of the remnant pancreas. We diagnosed it as a recurrence of the tumor and performed a total pancreatectomy for the remnant pancreas. In the histological evaluation of the resected specimen of the distal pancreas, the neoplastic cells formed an acinar and papillary structure that extended into the main pancreatic duct. Mucin5AC, α1-antitrypsin (α-AT) and carcinoembryonic antigen (CEA) were detected in the tumor cells by immunohistochemistry. In the resected head of the pancreas, the tumor was composed of both acinar and ductal elements with a mottled pattern. The proportions of each element were approximately 40% and 60%, respectively. Strongly positive α-AT cells were detected in the acinar element. Some tumor cells were also CEA positive. However, the staining for synaptophysin and chromogranin A was negative in the tumor cells. Ultimately, we diagnosed the tumor as a recurrence of mixed acinar-ductal carcinoma in the remnant pancreas. In conclusion, we report here a rare case of repeated pancreatic resection for multicentric lesions of mixed acinar-ductal carcinoma of the pancreas.

Key words: Mixed acinar-ductal carcinoma; Pancreatic cancer; Acinar carcinoma; Total pancreatectomy

Core tip: We report a rare case of multicentric mixed acinar-ductal carcinoma lesions of the pancreas. The patient lived for 39 mo after the first operation without a second recurrence.

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INTRODUCTION

Although several cases of mixed acinar-endocrine carcinoma of the pancreas, which is composed of both

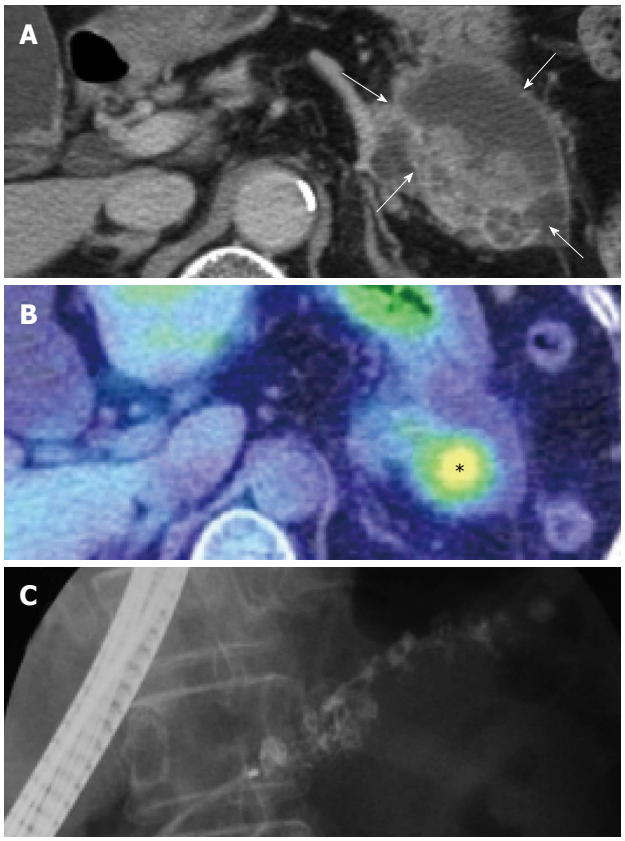

Figure 1 Diagnostic imaging before the first operation. A: Computed tomography showed a low density area (approximately 6 cm) in the pancreas tail (arrow); B: Positron emission tomography-computed tomography showed abnormal uptake of fluorodeoxy glucose (asterisk) in the low density area observed on computed tomography; C: Endoscopic retrograde cholangiopancreatography showed irregular dilatation of the main pancreatic duct in the body and tail.

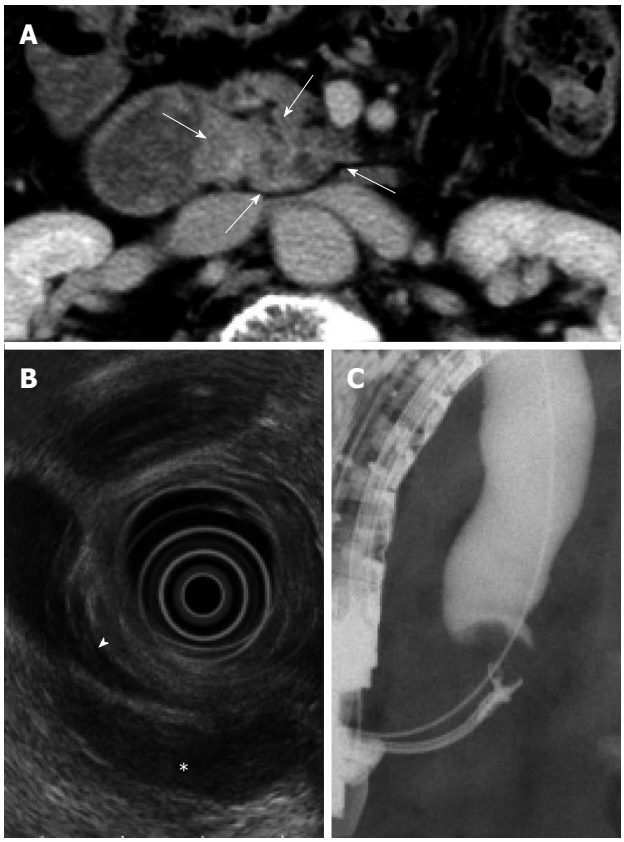

Figure 2 Diagnostic imaging before the second operation. A: Computed tomography showed a low density area at the uncus of the pancreas (arrow); B: Endoscopic ultrasound showed a 3 cm hypoechoic mass (asterisk) at the main pancreatic duct near the common bile duct (arrowhead); C: Endoscopic retrograde cholangiopancreatography showed the presence of inferior common bile duct stenosis.

acinar and endocrine tumor cells, have been reported[1], there have only been a few cases of resected mixed acinar-ductal carcinoma worldwide. According to the World Health Organization (WHO) classification, mixed acinar-ductal carcinoma is considered to be a sub-class of acinar cell neoplasms[2]. Mixed acinar-ductal carcinoma is defined in cases where greater than 25% of the tumor exhibits acinar and ductal elements in the pathological findings. Mixed acinar-ductal carcinoma usually exhibits an aggressive behavior, and it has a poor prognosis[3].

Herein we report a case of repeat pancreatectomy for multicentric lesions diagnosed as mixed acinar-ductal carcinoma, discuss the clinicopathological features, and present a review of the literature.

CASE REPORT

Clinical features

A 71-year-old female presented with epigastric pain in October 2009. Abdominal computed tomography (CT) showed a cystic lesion with a solid component in the tail of the pancreas (Figure 1A) and positron emission tomography-CT (PET-CT) showed intense fluorodeoxy glucose (FDG) uptake at the solid lesion (Figure 1B). Endoscopic retrograde cholangiopancreatography (ERCP) revealed irregular dilatation of the main pancreatic duct in the body and tail of the pancreas (Figure 1C). We diagnosed the patient with intraductal papillary mucinous neoplasms. During the operation, we found that the pancreas tumor was adhered to the duodenum, jejunum and transverse colon. We did not want to peel away the tumor, so we performed a distal pancreatectomy with splenectomy and partial resections of the duodenum, jejunum and transverse colon.

In March 2011 (15 mo after the first operation), the patient had did not exhibit any symptoms, but a follow-up CT examination showed a low density area at the uniciate process of the pancreas (Figure 2A). FDG/PET-CT revealed intense FDG uptake in the same region (data not shown). An endoscopic ultrasound examination showed a hypo-echoic lesion in the uniciate process of the pancreas that was approximately 3 cm in diameter, and this tumor compressed the inferior common bile duct (Figure 2B). ERCP revealed the presence of inferior common bile duct stenosis, and adenocarcinoma was suggested by a biopsy of the tumor lesion (Figure 2C). Based on these findings, we suspected a recurrence of mixed-acinar ductal carcinoma. Total resection of the remnant pancreas was performed. The patient was discharged successful on postoperative day 32. She re-

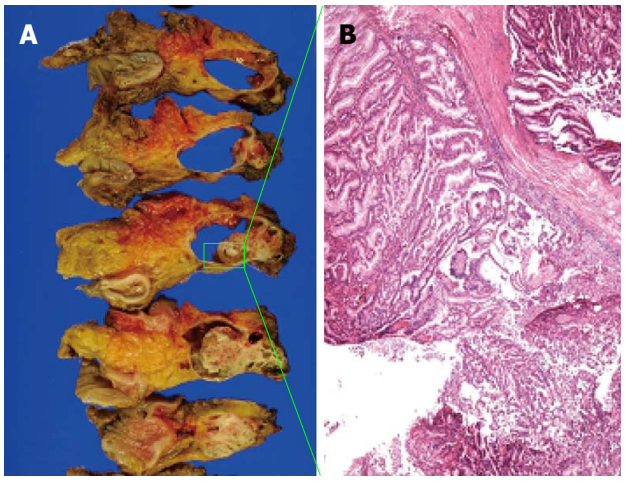

Figure 3 The resected specimen and pathological findings from the first operation. A: The resected specimen had a cystic lesion with a solid component; B: HE staining showed a mucus-type epithelium with an extension of intraductal papillary growth of the tumor cells. Scarce mucus-producing cells were also present (HE, × 40).

mained alive 21 mo after the second operation and had no recurrence.

Gross and pathological findings

During the distal pancreatectomy, a cystic lesion that was 7.2 cm in diameter with a partial solid component was noted in the specimen (Figure 3A). Hematoxylin and eosin (HE) staining showed both papillary and acinar-like structures in the solid component that extended into the main and the branch pancreatic duct (Figure 3B). In the former region, cube-shaped or high columnar cells exhibiting with mucin production formed the papillary structure resembling intraductal papillary neoplasms. Acinar-like structures were also observed in certain areas. No malignant cells were detected in the proximal stump of the pancreas. The tumor was localized to the pancreas without invasion into the duodenum, jejunum or, colon. We used an EnVision™ FLEX system for immunostaining. We assessed the ductal and acinar elements using immunohistochemistry. The results showed, positive staining for α1-antitrypsin (α-AT) and carcinoembryonic antigen (CEA). The gastrointestinal element was negative for mucin 2 (MUC2). Mucin 5AC (MUC5AC) was minimally detected in the tumor cells; however the we thought intraductal papillary mucinous neoplasm was negative (Figure 4). The islet element was negative for synaptophysin and chromogranin A (data not shown). Based on these histological findings, we diagnosed the tumor as a mixed acinar-ductal carcinoma. Extended papillary growth outside the cystic lesion was also observed. A total of 8 lymph nodes were extracted, but no metastasis of the resected lymph nodes was detected. The pathological stage was ⅢⅠb (T3N0M0) based on the 7th edition criteria of the Union for International Center Control[4].

The specimen taken during the second operation revealed another tumor approximately 3 cm in diameter tumor in the head of the pancreas (Figure 5A). Acinar-like structures and papillary growths were observed, with a mottled pattern were observed(Figure 5B). Some regions of the tumor had a papillary growth pattern (Figure 5C).

Some of the tumor cells with mild atypia showed poor mucus production with eosinophilic cytoplasm. The tumor exhibited a papillary proliferation pattern, with delicate fibervascular stroma and acinar-like structure pattern in some regions (Figure 5D). The tumor exhibited an almost solid growth, and the glandular lumen was unclear. We detected abundant mitotic figures in the tumor. In the microscopic findings, approximately 40% of the tumor had an acinar-like structure, and 60% of it exhibited a papillary growth pattern. There was intense α-AT staining in the acinar-like part of the tumor. MUC5AC was not detected. Mild positive staining for mucin1 and CEA was observed (Figure 6). The staining for synaptophysin A and chromogranin were negative (data not shown). The tumor showed both an acinar-like and papillary growth pattern in the main pancreatic duct. As a result, we diagnosed the tumor as a mixed acinar-ductal carcinoma in the remnant pancreas. A total of 7 lymph nodes were extracted, but metastasis was not detected.

DISCUSSION

Combined acinar and ductal phenotype carcinoma of the pancreas is very rare[5-7]. The WHO classification categorizes mixed acinar-ductal pancreatic carcinoma as a sub-class of acinar cell neoplasms[2]. Mixed carcinomas of the pancreas have distinctive histological features suggesting more than one line of differentiation. Mixed acinar-ductal carcinomas are defined as those in which at least 25 percent of the neoplastic cells show an acinar and ductal line of differentiation[2,8].

Stelow *et al*[3] reported a study of 11 cases of mixed acinar-ductal carcinoma. In their report, all cases showed significant evidence of acinar and ductal differentiation that were, estimated to include at least 25% of the neoplastic cells. In the present case, acinar differentiation was shown to involve 40% of the tumor, with the remaining portion exhibiting ductal differentiation during the second operation. All but one of the carcinomas showed predominantly acinar differentiation, based on routine histological and immunohistochemical analyses[3]. The 18 previously reported cases of mixed-acinar ductal carcinoma in the English and Japanese literatures are summarized in Table 1[3,9-11]. In five cases, the tumors were present in the tail of the pancreas, while in the remaining cases, the tumors were located in the head of the pancreas (the tumor diameters ranged from 35-72 mm). With regard to treatment, only one case received radiotherapy, and the other cases underwent surgical resection. There were no reports of a recurrence of the tumor or additional resection of the remnant pancreas.

Kobayashi *et al*[12] reported an intraductal growth of tumor cells into the main pancreatic duct with acinar endocrine carcinomas. For this reason, it is possible that mixed acinar and ductal phenotype carcinoma is biologi-

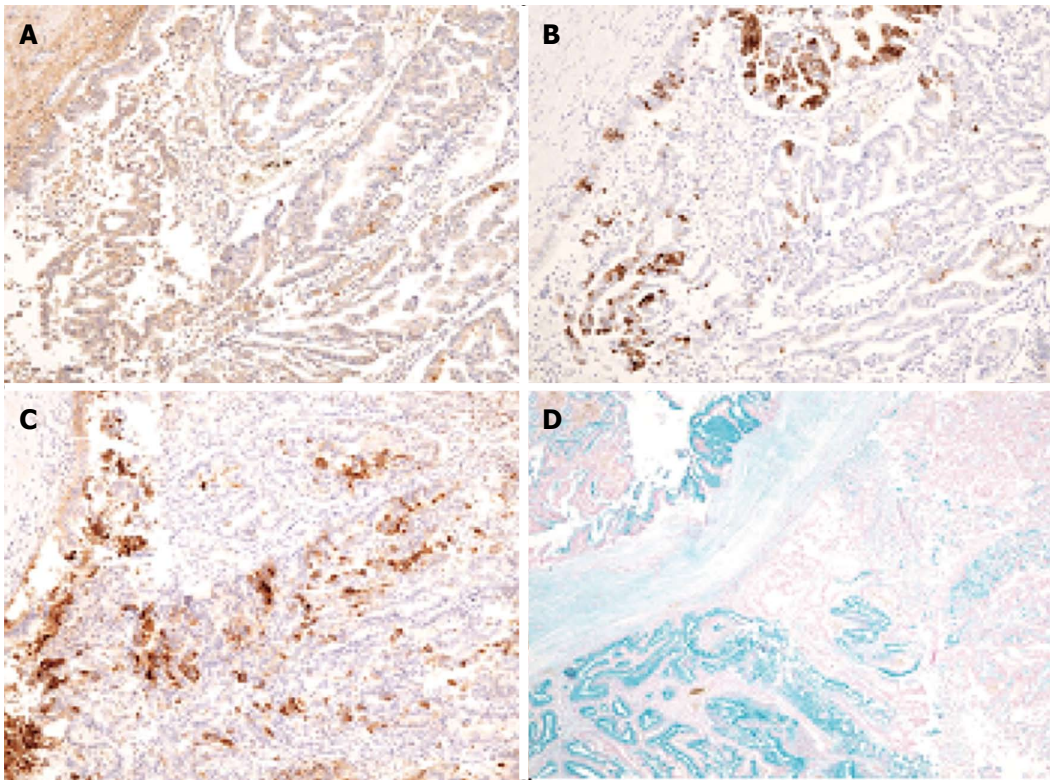

Figure 4 Immunohistochemical and pathological findings from the first operation. A: α1-antitrypsin (× 100): regions of both strongly positive and weakly positive cells were present; B: Mucin5AC (× 100): only part of the tumor was positive; C: Carcinoembryonic antigen (× 100): a portion of the tumor was positive; D: Alcian blue (× 100): staining was positive in the region with poor mucus production.

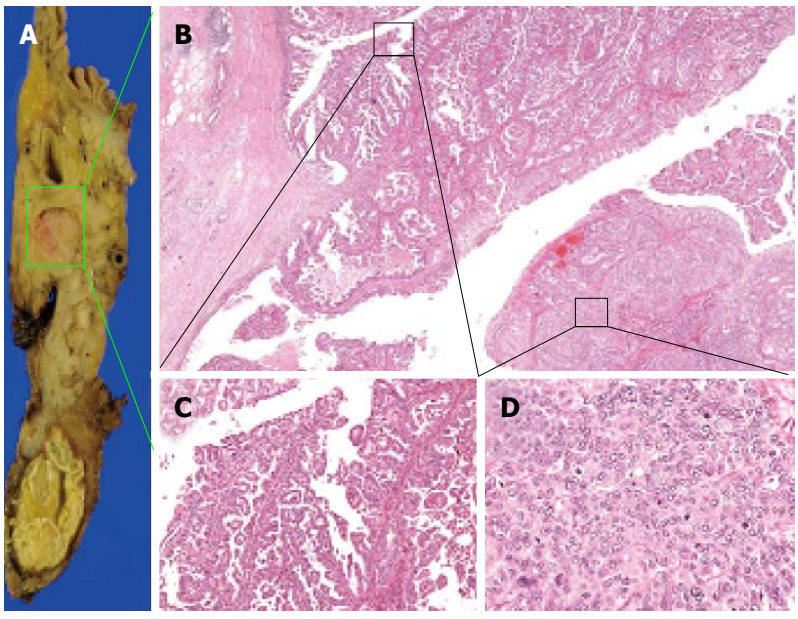

Figure 5 The resected specimen and pathological findings from the second operation. A: The resected specimen from the second operation showed a 3 cm solid mass in the head of the pancreas; B: There was a region with acinar-like structures (bottom right) that showed papillary growth (upper left) (HE, × 40); C: Dysplastic cells with poor mucus production exhibited papillary proliferation (HE, × 200); D: Acinar structures and cell division were observed (HE, × 400).

cally more closely related to acinar cell carcinoma than to pancreatic duct carcinoma. Five of the previously reported cases had a "mucinous acinar cell carcinoma" pattern that, which is characterized by the production of mucin in the acinar cell carcinoma. Six cases were reported to exhibit a "combined acinar ductal" pattern which finds mottled component of acinar cell carcinoma and ductal carcinoma. Our case was a "mucinous acinar cell carcinoma" at the first operation and a "combined acinar ductal carcinoma" at the second operation[3].

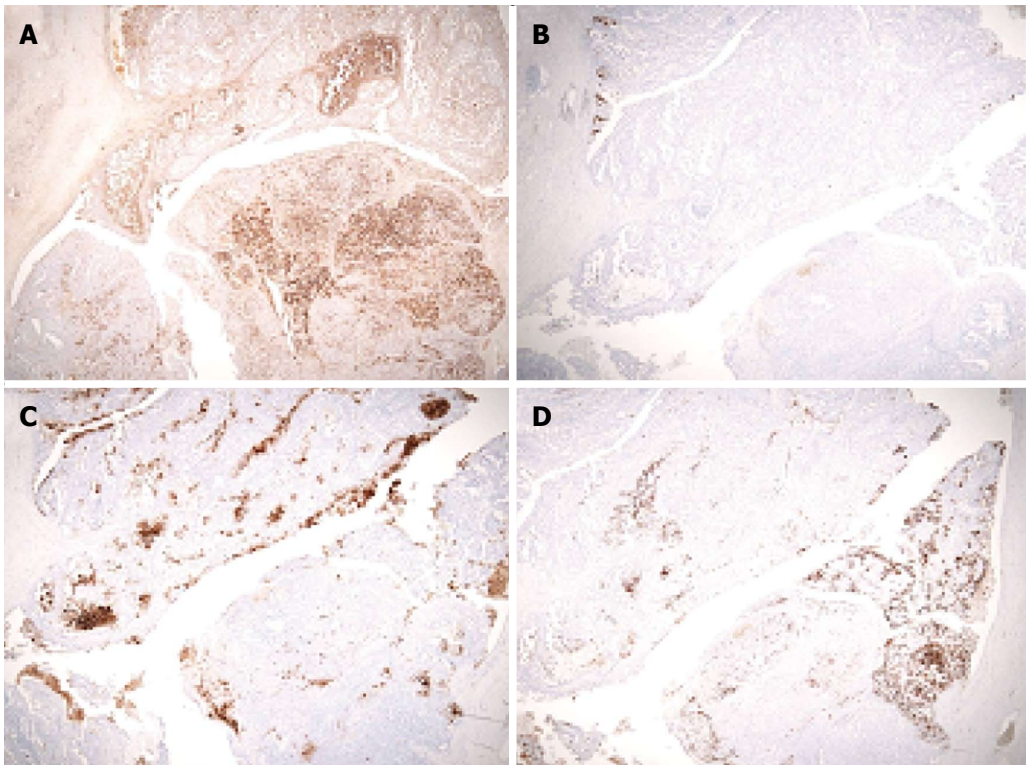

Figure 6 Immunohistochemical and pathological findings from the second operation. A: α1-antitrypsin (× 40): the acinar structure in the lower right area was strongly positive; B: Mucin5AC (× 40): only a small part of the tumor was positively stained; C: Carcinoembryonic antigen (× 40): only a portion of the area was positively stained; D: Mucin1 (× 40): some positive staining was observed.

Table 1 The 18 previously reported cases of mixed-acinar ductal carcinoma in the English and Japanese literatures

Case	Age	Sex	Symptoms at presentation	Size	Location	Treatment	Follow-up	Prognosis	Reference
1	74	M	Painless jaundice	31	Head	RTx, CTx	20	Alive	[3]
2	75	M	Weight loss and diarrhea	25	Head	RTx, CTx	39	Dead	[3]
3	73	M	Not available	20	Tail	RTx, CTx	52	Dead	[3]
4	74	M	Weight loss and diarrhea	40	Head	RTx, CTx	51	Dead	[3]
5	70	M	Pain	40	Head	RTx, Rdx	38	Dead	[3]
6	77	F	Weight loss	30	Head	Rdx	9	Dead	[3]
7	77	M	Weight loss and pain	37	Head	RTx	0.5	Dead[1]	[3]
8	52	M	Pain	55	Head	RTx, CTx	12	Dead	[3]
9	76	M	Painless jaundice	35	Head	RTx, CTx	8	Dead	[3]
10	79	M	Painless jaundice	34	Head	RTx, CTx	11	Alive	[3]
11	69	M	Painless jaundice	54	Head	RTx, CTx	36	Alive	[3]
12	71	M	Not mentioned	30	Head	-	12	Dead	[10]
13	51	F	Not mentioned	-	Tail	-	3	Dead	[10]
14	51	F	Not mentioned	30	Tail	-	4	Dead	[10]
15	85	M	Not mentioned	-	Tail	-	6	Dead	[10]
16 (J)	63	M	Abdominal pain	65	Head	RTx	39	Alive	[11]
17 (J)	63	M	Worsening of diabetes	35	Head	RTx	8	Dead	[12]
18	71	F	Epigastric discomfort	72	Tail	RTx	36	Alive[2]	
				35	Head	RTx			

[1]Deceased due to a post operative complication; [2]Recurrence in the remnant pancreas 18 mo after the first operation. J: Japanese article; M: Male; F: Female; RTx: Resection; Rdx: Radiation; CTx: Chemotherapy; -: Not mentioned.

Mucin production was observed in all cases, with a high positive rate of immunostaining for MUC1 and CEA that, was useful in the diagnosis. This was also the case consistent with our patient. The clinical course was considered to be aggressive, and seven of the previous patients died at a mean of 29 mo after the operation[3]. However, our patinet remained alive without recurrence 39 mo after the first operation.

Our patient underwent repeated pancreatectomy due to a metachronous mixed acinar-ductal carcinoma. A metachrounous tumor in the remnant pancreas of mixed acinar-ductal carcinoma has not previously been reported,

although repeated pancreatectomy for some cases of pancreatic duct carcinoma, intraductal papillary, mucinous tumors and endocrine tumors has[13-18]. There was only one previous case of a recurrence of acinar carcinoma[14].

In conclusion, we reported a rare case of mixed acinar-ductal carcinoma. The patient developed metachrounous tumors in the remnant pancreas and underwent a repeated resection. She remained alive 39 mo after the first operation without a second recurrence.

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CASE REPORT

Novel method to prevent gastric antral strictures after endoscopic submucosal dissection: Using triamcinolone

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Abstract

Endoscopic submucosal dissection (ESD) of large gastric lesions often leads to severe gastric strictures, especially in cases of large ESD in the antrum of the stomach. It has recently been reported that balloon dilation, mucosal incision, and local steroid injections can successfully treat gastric strictures. However, there are some complications with existing methods and decreasing the quality of life. We have developed a novel method to prevent severe gastric strictures that does not involve balloon dilation, mucosal incision, or steroid injections after circumferential ESD. Our original method involves the submucosal injection of a mixed solution composed of triamcinolone acetonide and a general solution of glycerol, hyaluronic acid, and a small amount of indigo carmine and epinephrine during the ESD procedure; this mixture is called a mixed solution of triamcinolone (MST). According to standard ESD procedures, several milliliters of MST are injected into the submucosal layer for the purpose of elevating the submucosa during ESD resulting in prevention of severe strictures. Our method using MST take several advantages such as MST method suppress inflammation in ulcer from initial phase, prevention of stricture without obstructive symptoms, and does not require several ballooning. Therefore, MST method is safe and gentle, shorten the hospitalization duration. Here, we described two cases in which we prevented severe strictures of the gastric antrum after completing a circumferential ESD using MST without any complications.

Key words: Early gastric cancer; Endoscopic submucosal dissection; Stricture of antrum; Triamcinolone

Core tip: Endoscopic submucosal dissection (ESD) leads to severe gastric strictures, especially in cases of large ESD in the antrum of the stomach. It has recently been reported that some methods can treat gastric strictures, however there are some limitations. We have developed a novel method to prevent severe gastric strictures that does not involve previous methods. Our original method involves the submucosal injection of a mixed solution composed of triamcinolone acetonide and a general solution during the ESD procedure. Here, we described two cases in which we prevented severe strictures of the gastric antrum after ESD using our original method without any complications.

Nishiyama N, Mori H, Kobara H, Rafiq K, Fujihara S, Matsunaga T, Ayaki M, Yachida T, Oryu M, Masaki T. Novel method to prevent gastric antral strictures after endoscopic submucosal dissection: Using triamcinolone. *World J Gastroenterol* 2014;

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INTRODUCTION

Endoscopic submucosal dissection (ESD) was developed to enable the *en bloc* removal of large gastric cancers while decreasing the risk of local recurrences and allowing the collection of sufficient specimens for an accurate histopathological evaluation. The ESD procedure is associated with a high incidence of stenosis in both the esophagus and stomach. In addition, strictures after ESD have frequently been described in esophageal lesions[1,2]. However, so far, there have been only a few reports of strictures after ESD in the stomach[3-8]. Previous it has been reported that the extent of a circumferential mucosal defect of > 3/4 in both the antrum and cardia is a relevant factor for gastric stenosis after ESD[6-8]. We have treated the cases of severe stenosis in the antrum after ESD, and the circumferential extents of the mucosal defects were > 3/4 (Figure 1A-C). In such cases, we have had to rely upon endoscopic balloon dilation[3,6-8] and local steroid injection[4,5]. Recently, the efficacy of balloon dilation and local steroid injection for the prevention and treatment of post-ESD strictures has been described for the esophagus and stomach[1-8]. However, several additional endoscopic procedures after ESD were required when using such methods resulting in additional costs, patient inconveniences, and increased rates of perforation[3]. Therefore, we considered a new method involving submucosal injection of a mixed solution composed of triamcinolone acetonide (Kenacort: 50 mg/5 mL; Bristol-Meyers Squibb Co. Tokyo, Japan) 8 mL, glycerol 16 mL, hyaluronic acid 16 mL, and a small amount of indigo carmine and epinephrine: this mixture is to be administered during the ESD procedure. This mixture is called a mixed solution of triamcinolone (MST). The lesions were treated with standard ESD procedure using a dual knife (KD-650L; Olympus Co., Tokyo, Japan) and an IT2 knife (KD-611L; Olympus Co., Tokyo, Japan). According to the standard ESD procedure, several milliliters of MST was injected into the submucosal layer just beneath the lesion for the purpose of elevating the submucosa. Thus, we were able to equally permeate the steroids into the remaining submucosa of the dissected ulcer defect (Figure 2).

This mixed steroid injection method was able to suppress inflammation at early phase immediately after ESD procedure. We treated two patients with mucosal gastric cancer using circumferential ESD in the antrum. The circumferential extents of the mucosal defects were > 3/4 in the antrum of the stomach. We administered MST injections into the submucosal layer as treatment. Two months later, there were no severe strictures or gastric outlet obstructions after ESD procedure, even in the absence of balloon dilation or further steroid injections.

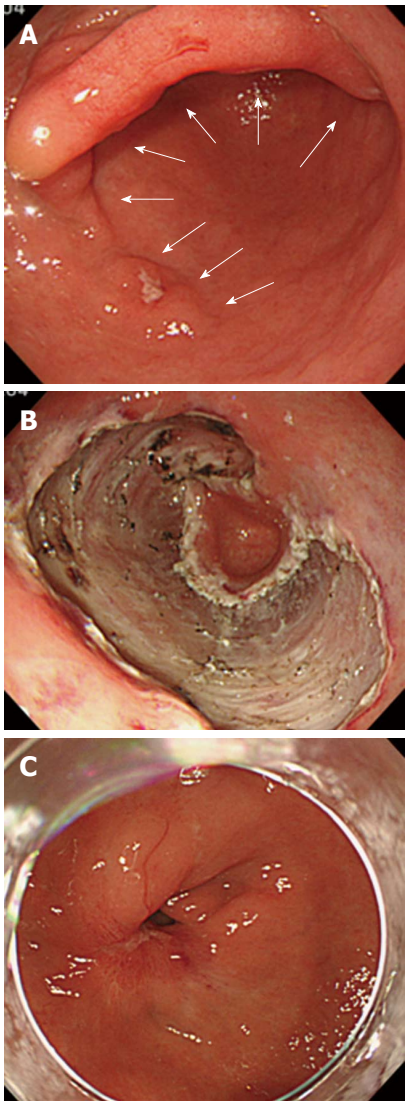

Figure 1 We treated a case of severe stenosis in the antrum after the endoscopic submucosal dissection procedure. The circumferential extent of the mucosal defect was > 3/4. A: The white arrow shows that the lesion constituted of a semi-circumferential, laterally spreading type 0-IIc early gastric cancer on the anterior wall of the antrum; B: The circumferential extent of the mucosal defect was > 4/5 after the performance of the ESD procedure; C: Three months after the endoscopic submucosal dissection operation, the patient experienced a severe stricture of the gastric antrum. Subsequently, we had to perform a mucosal incision and local steroid injection.

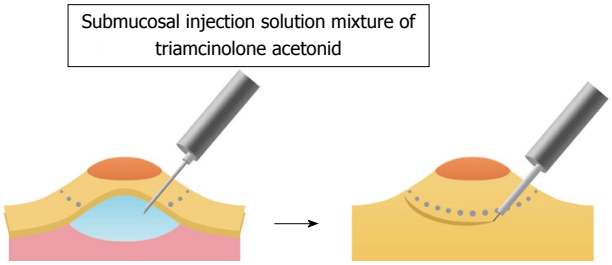

Figure 2 Schematic diagram of our proposed new method. Schematic diagram of our proposed new method consists of submucosal injection of a mixture of solution containing triamcinolone acetonide 8 mL, glycerol 16 mL, and hyaluronic acid 16 mL, in addition to a small amount of indigo carmine and epinephrine, which was injected during the performance of the endoscopic submucosal dissection (ESD). The lesions were treated by standard ESD procedures.

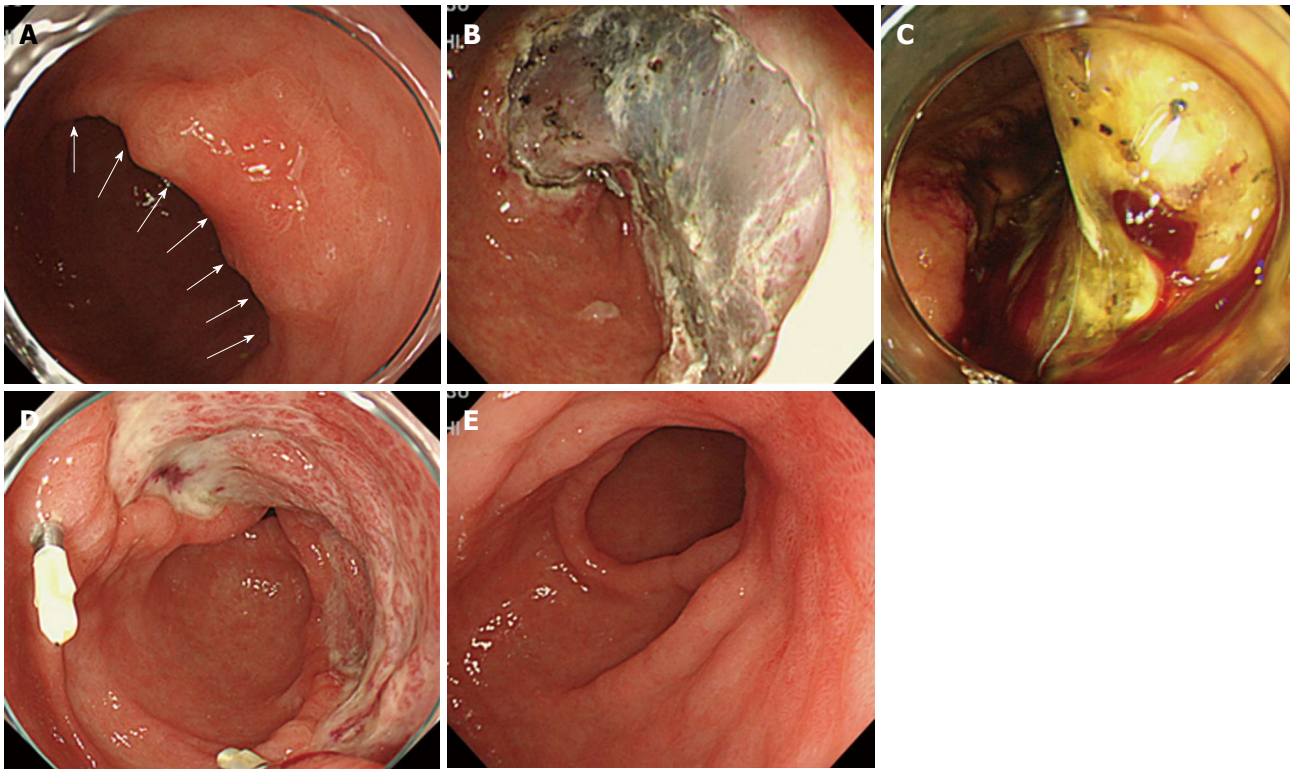

Figure 3 We performed our proposed new method during endoscopic submucosal dissection at gastric antrum (Case 1). A: Endoscopic imaging showed that the lesion constituted a semi-circumferential, laterally spreading type 0-IIc early gastric cancer in the posterior wall of the antrum (white arrow); B: A three-fourths circumferential mucosa in the antrum was removed by standard endoscopic submucosal dissection (ESD) procedure; C: The day after the ESD operation, there was abundant vascularization in the ESD ulcer; D: Two weeks after the ESD operation, there was more granulation tissue than in a typical ESD ulcer; E: Three months after the ESD operation, there were no strictures concomitant with the healing process near the ESD ulcer.

CASE REPORT

Case 1

A 63-year-old man was admitted to our hospital for early gastric cancer diagnosed by a previous esophagogastroduodenoscopy (EGD) examination. The lesion was a semi-circumferential, laterally spreading type 0-Ⅱc early gastric cancer on the posterior wall of the antrum (Figure 3A). We assumed that the circumferential extent of the mucosal defect would be > 3/4 in the antrum of the stomach after ESD. Informed consent was obtained from the patient before ESD operation as required by our Hospital Clinical Ethics Committee. The lesion was initially treated by the standard ESD procedures. During ESD, a mixture of triamcinolone acetonide 8 mL, glycerol 16 mL, hyaluronic acid 16 mL, and a small amount of indigo carmine and epinephrine was injected into the submucosal layer just beneath the lesion.

The total dose of triamcinolone acetonide injection was 60 mg. Three-fourths circumferential section of mucosa in the antrum was removed (Figure 3B). The day after the ESD operation, there was abundant vascularization in the ulcer area more than in patients with a normal ESD procedure without MST injection (Figure 3C). Two weeks after the ESD operation, there was more granulation tissue than usual in ESD ulcers site (Figure 3D). Three months after the ESD operation, the ulcer entered into a healing stage, and no strictures developed concomitant with the healing process near the ESD ulcer site (Figure 3E). No complications, including nausea or vomiting developed following the ESD procedure.

Case 2

A 61-year-old man was admitted to our hospital for early gastric cancer diagnosed by a previous EGD examination. The lesion was a one-third circumferential, laterally spreading type 0-Ⅱc early gastric cancer on the anterior wall of the antrum (Figure 4A). We predicted that the circumferential extent of the mucosal defect would be > 3/4 in the antrum of the stomach after ESD. After obtaining informed consent, we used the same steroids as those used in Case 1 during the ESD. The total injected dose of triamcinolone acetonide was 40 mg. A three-fourths circumferential mucosa in the antrum was removed (Figure 4B). The day after the ESD, there was more abundant vascularization in the ulcer than usual (Figure 4C). Two weeks after the ESD operation, there was also more granulation tissue than usual in ESD ulcers site (Figure 4D). Three months after the ESD operation, the ulcer had entered a healing stage, and there were no strictures concomitant with the healing process near the ESD ulcer (Figure 4E). No nausea or vomiting occurred during these three months, and no other complications occurred following the ESD procedure.

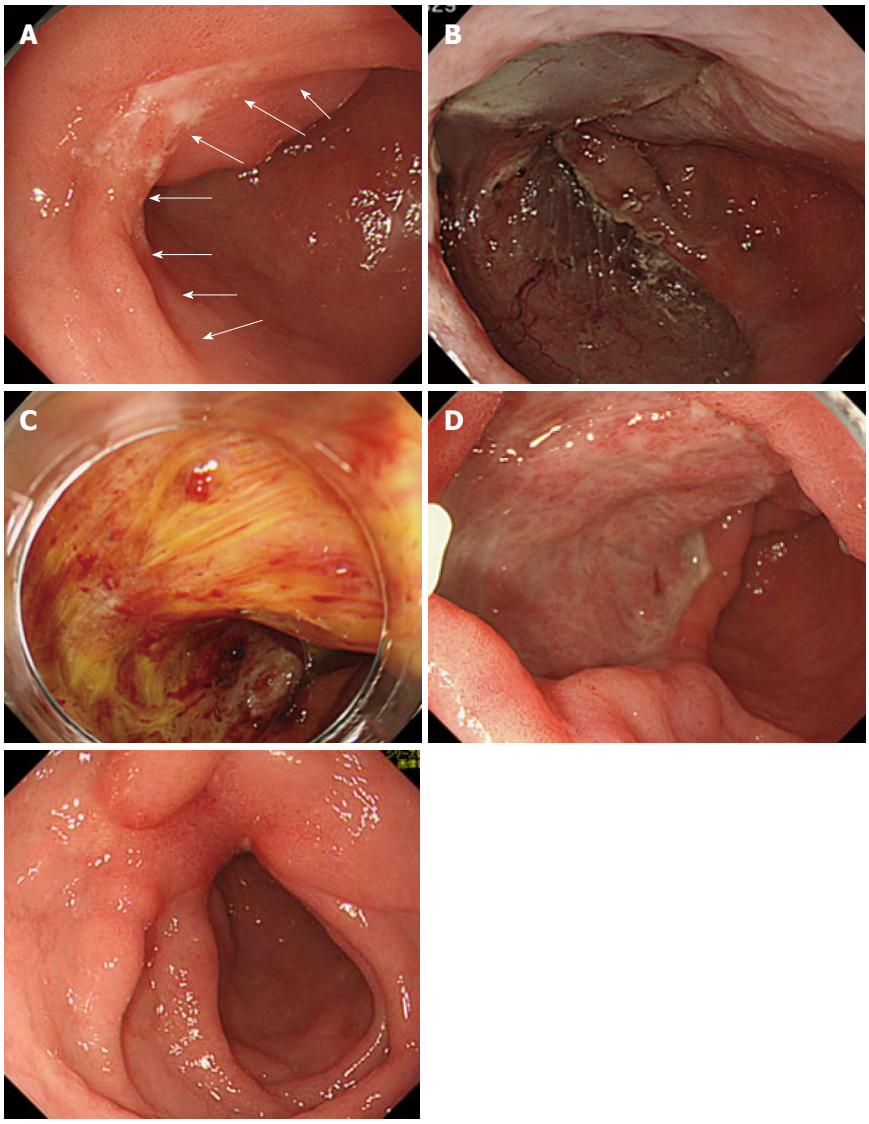

Figure 4 We performed our proposed new method during endoscopic submucosal dissection at gastric antrum (Case 2). A: Endoscopic imaging showed that the lesion constituted a one-third circumferential, laterally spreading type 0-IIc early gastric cancer on the anterior wall of the antrum (white arrow); B: A three-fourths circumferential mucosa in the antrum was removed by standard endoscopic submucosal dissection (ESD) procedure; C: The day after the ESD, there was abundant vascularization in the ESD ulcer; D: Two weeks after the ESD operation, there was more granulation tissue than usual in the ESD ulcer; E: Three months after the ESD operation, there were no strictures concomitant with the healing process near the ESD ulcer.

DISCUSSION

Based on our initial experience with injecting MST during ESD procedure, this method appears to be effective for preventing severe gastric strictures without balloon dilation, mucosal incision, or steroid injections after circumferential ESD treatment.

It has been reported that steroid injections can prevent strictures after ESD for esophageal cancer and can lower the number of endoscopic balloon dilation sessions[1]. ESD scar formation is thought to be an integral part of wound healing, a process that involves inflammation, proliferation, and remodeling. Strictures develop due to the crosslinking of collagen. Steroid injections can induce the regression of scars by suppressing inflammation and reducing collagen and fibroblast proliferation. Because the inflammatory process starts immediately after the ESD procedure, it is necessary to use steroids as soon as possible to suppress excess inflammation[1,2,5,9,10].

One advantage of our novel method is that it allows us to inject steroids into the submucosal layer just beneath the lesion during the ESD procedure. This direct application reduces the level of inflammation resulting from the initial phase. Previous reports have indicated that balloon dilation can treat strictures of the gastric antrum that develop after circumferential ESD treatment is performed[3,6-8]. However, out of four patients who underwent a step-series endoscopic balloon dilatation, two of them experienced gastric perforation (50% incidence of perforation)[3]. Therefore, balloon dilation may be one option, but its use should be limited due to its associated complications. Moreover, ballooning methods must be performed several times after the ESD, resulting in a longer hospitalization time due to the serial

Table 1 Advantages and disadvantages of mixed solution of triamcinolone method with other methods

Methods	Advantages	Disadvantages
Balloon dilatation	Technical easy	Perforation
		Longer hospitalization
		Require several endoscopies
		Treatable after obstructive symptoms
Incision and steroid injection	Suppress inflammation of ulcer	Technical difficult
	Safe	Longer hospitalization
		Treatable after obstructive symptoms
Mixed solution of triamcinolone method	Suppress inflammation of ulcer from initial phase	Delayed bleeding (abundant vascular)
	Shorter hospitalization	
	Safe and gentle	
	Prevention of stricture	

endoscopies that must be performed and decreasing the patient's quality of life. As our MST method does not require ballooning, therefore it is safer and gentler than the ballooning method. In fact, our two patients were only hospitalized for 7 d following the ESD procedure, a length similar to that for standard ESDs. We also summarized possible advantages and disadvantages of MST method with other existing methods in Table 1.

Recently, our colleague reported that mucosal incisions and local steroid injections were effective in treating stenosis of the gastric antrum after circumferential ESD[4]; we also consider this approach effective. However, this method has challenges associated with the injection of triamcinolone into the submucosal layer within the ulcer after the ESD and is also accompanied by the possibility of perforations, similar to esophageal cases[11]. Thus, to address these issues, we developed the present MST procedure, which is more gentle and convenient than previous methods.

Furthermore, previous methods have been able to treat strictures only after complaints of obstructive symptoms or after severe stricture have occurred, while our MST can prevent strictures in the antrum after circumferential ESD. Although the use of steroids delays the healing of ulcers after ESD, they minimize severe deformities and strictures. However, due to the induction of abundant vascularization, as shown in our two cases (Figures 3C, 4C); care must be taken to detect any delayed bleeding for at least 7 d after ESD procedure.

Additional experience with our method is still needed. Currently, a comparative prospective study including a large number of cases is on-going at our hospital.

If the circumferential extent of a mucosal defect is > 3/4 at the antrum of the stomach after ESD procedure, MST injection during ESD procedure is to be an effective and convenient method to prevent strictures without steroid injection alone and performance of ballooning.

COMMENTS

Case characteristics

Case-1 was a 63-year-old man was admitted to our hospital for early gastric cancer, who had a semi-circumferential, laterally spreading type 0-Ⅱc early gastric cancer on the posterior wall of the antrum. Case-2 was a 61-year-old man was admitted to our hospital for early gastric cancer, who had a one-third circumferential, laterally spreading type 0-Ⅱc early gastric cancer on the anterior wall of the antrum.

Treatment

Authors have developed a novel method to prevent severe gastric strictures that does not involve previous methods. Their original method involves the submucosal injection of a mixed solution composed of triamcinolone acetonide and a general solution during the Endoscopic submucosal dissection (ESD) procedure.

Related reports

It has recently been reported that balloon dilation, mucosal incision, and local steroid injections can successfully treat gastric strictures. However, there are some complications with existing methods and decreasing the quality of life.

Term explanation

Their original method involves the submucosal injection of a mixed solution composed of triamcinolone acetonide and a general solution of glycerol, hyaluronic acid, and a small amount of indigo carmine and epinephrine during the ESD procedure; this mixture is called a mixed solution of triamcinolone (MST).

Experiences and lessons

If the circumferential extent of a mucosal defect is > 3/4 at the antrum of the stomach after ESD procedure, we consider MST to be an effective and convenient method to prevent strictures without steroid injection alone and performance of ballooning.

Peer review

Additional experience with this method would be needed to find out any severe side-effects in clinic.

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CASE REPORT

Two cases of neuroendocrine carcinoma of the gallbladder

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Abstract

Neuroendocrine carcinoma (NEC) of the gallbladder is a rare subtype of gallbladder tumor. Here, we report two cases of NEC in two patients initially suspected to have gallbladder carcinoma. No specific symptoms or abnormal blood test results were observed preoperatively. Abdominal computed tomography scans indicated intraluminal masses in the gallbladder and lymph node enlargement in the hepatic hilum. Radical cholecystectomy and regional lymphadenectomy were performed. The first patient also presented with liver invasion and therefore underwent resection of liver segment Ⅳ. A diagnosis of NEC was made upon postoperative pathological examination and immunohistochemical staining according to the WHO Classification of Tumors of the Digestive System (2010). One tumor was identified as poorly differentiated NEC and the other as poorly differentiated mixed adenoneuroendocrine carcinoma. Immunohistochemical staining data from both tumors showed positivity for chromogranin A and synaptophysin. The first patient received 4 cycles of chemotherapy consisting of cisplatin and etoposide. No metastases or recurrence were observed 12 mo following surgery. The second patient refused chemotherapy and presented with tumor recurrence 4 mo after surgery. In conclusion, NEC of the gallbladder is an aggressive tumor and the identification of a standardized optimal treatment still requires further research. Our experience together with published studies suggests that radical surgery and adjuvant chemotherapy may improve the prognosis.

Key words: Neuroendocrine carcinoma; Tumor; Gallbladder; Chemotherapy; Carcinoid

Core tip: The authors report two cases of neuroendocrine carcinoma of the gallbladder, which is an extremely rare disease. Both patients received radical surgery and showed responses to chemotherapy. The authors discuss possible approaches to improving prognosis including surgery, chemotherapy, radiotherapy and biological targeted therapy in the context of their experience and previously published studies.

Chen H, Shen YY, Ni XZ. Two cases of neuroendocrine carcinoma of the gallbladder. *World J Gastroenterol* 2014; 20(33): 11916-11920 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i33/11916.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i33.11916

INTRODUCTION

Neuroendocrine tumors (NETs) are neoplasms originating from neuroendocrine cells located throughout the body, most commonly in the lung and gastrointestinal tract[1,2]. NETs are generally subclassified by site of origin and histological characteristics including tumor differentiation and grade. Neuroendocrine carcinoma (NEC) often refers to tumors with poor differentiation and high grade. Well differentiated tumors are defined as carcinoid and can further be divided into typical and atypical, based on the grade or biological behavior[3]. NETs of the gallbladder are rare and account for only 0.5% of all NETs[4] and

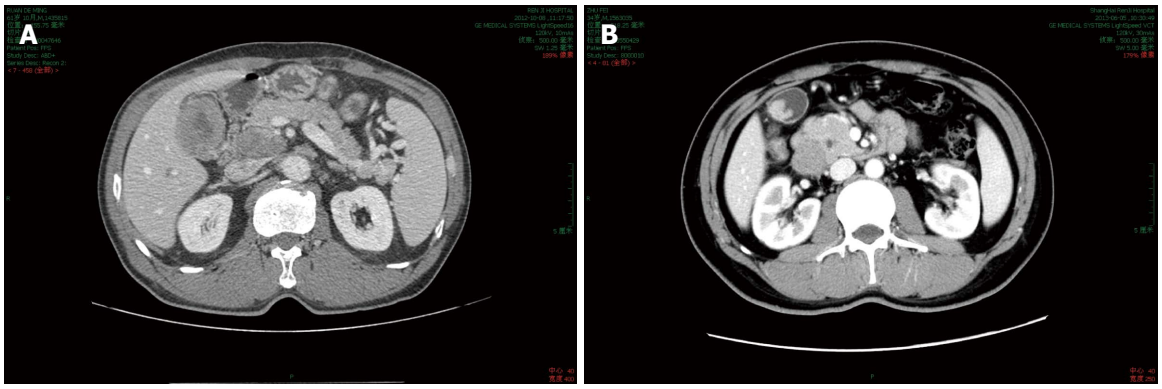

Figure 1 Computed tomography scan of the gallbladder carcinoma. A: A thick-walled gallbladder with local infiltration to the adjacent liver; B: A polypoid intraluminal lesion about 4 cm in diameter.

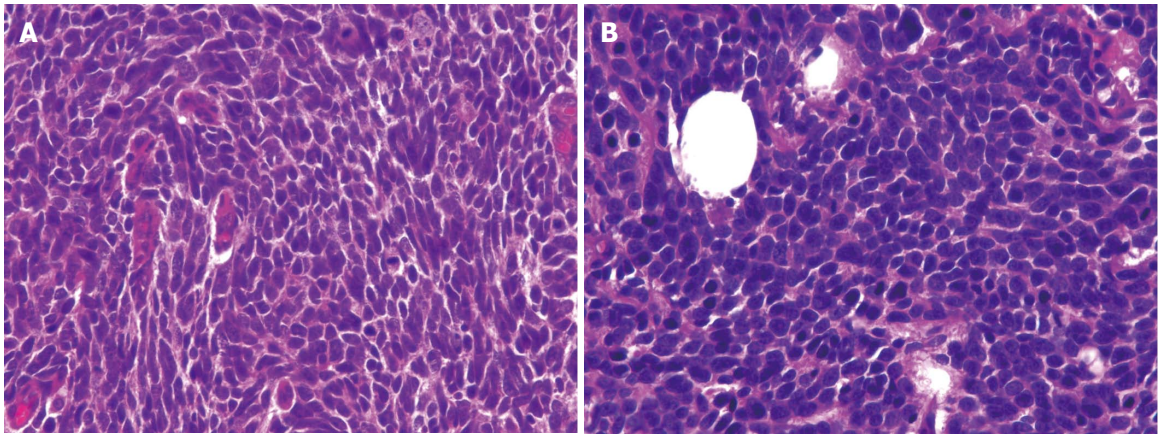

Figure 2 Pathological examination, HE, × 400. A: Postoperative pathological findings were poorly differentiated neuroendocrine carcinoma with serosal invasion and liver invasion; B: No transitional areas were observed between the two different parts.

2% of all gallbladder tumors, as reported by Yao *et al*[2]. Among 278 cases of NETs of the gallbladder reported in the Surveillance, Epidemiology, and End Results database, only five well-differentiated NETs were registered[5]. Almost all patients with NEC of the gallbladder are diagnosed incidentally on the basis of pathological examination and postoperative immunohistochemical staining[6]. Because of the relative paucity of cases, the clinicopathological characteristics and prognoses for NEC of the gallbladder remain largely undetermined. Here we report two cases of gallbladder tumor, one case of NEC and one of mixed adenoneuroendocrine carcinoma (MANEC). Both patients underwent radical surgery and showed good responses to chemotherapy consisting of cisplatin and etoposide.

CASE REPORT

Case 1

A 62 year-old male presented to our hospital with a history of nausea and vomiting for 2 wk. He had no abdominal pain, jaundice, weight loss or other complaints. The physical examination was normal. Full blood count, renal and liver function tests were within normal ranges. Because of vomiting, his serum sodium and chlorine concentrations were moderately decreased to 122 mmol/L and 85 mmol/L, respectively. Levels of tumor markers were as follows: AFP 2.88 ng/mL (normal range 0-7 ng/mL), carcinoembryonic antigen (CEA) 81.23 μg/L (normal range 0-4.8 μg/L), 19-9 carbohydrate antigen (CA19-9) 31.52 μg/mL (normal range 0-33 μg/mL). Contrast-enhanced computed tomography (CT) depicted a thick-walled gallbladder with local infiltration to the adjacent liver and no gallstones (Figure 1A). There were no signs of distal metastases in CT scans of the chest, abdomen and pelvis. The patient had a 3 year history of hypertension and denied any family history of cancer. He had undergone appendectomy 12 years before and endoscopicpolypectomy of the colon 2 years before. A solid mass of 4 cm × 4 cm × 2.5 cm in size was found in the body of the gallbladder, infiltrating the liver parenchyma for about 1 cm. En bloc cholecystectomy, resection of liver segment IV and regional lymphadenectomy were performed. Postoperative pathological findings (Figure 2A) were poorly differentiated NEC with serosal invasion and liver invasion. Immunohistochemical staining revealed positive expression of chromogranin A (CGA) (Figure 3A), synaptophysin (SYN) (Figure 4A), cytokeratin (CK) and cluster of differentiation (CD) 56. Furthermore, the Ki-67 index was over 80%. The level of CEA

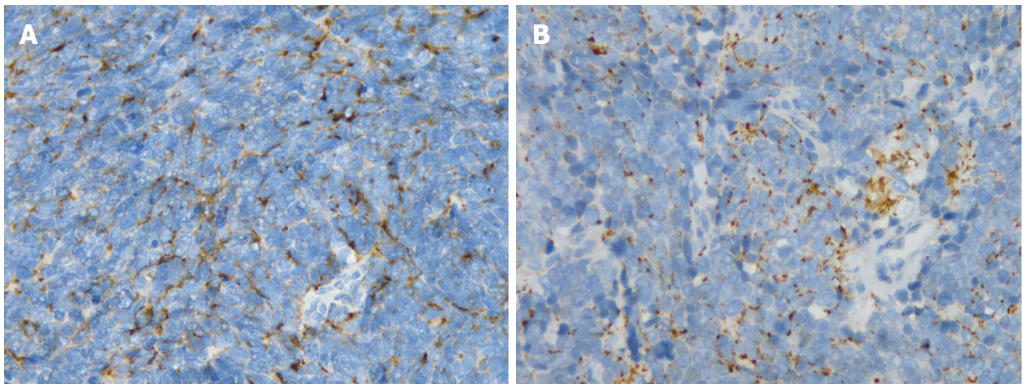

Figure 3 Immunohistochemical staining for chromogranin A. A: Immunohistochemical staining revealed positive expression of chromogranin A (CGA); B: Immunohistochemically, the tumor was positive for CGA.

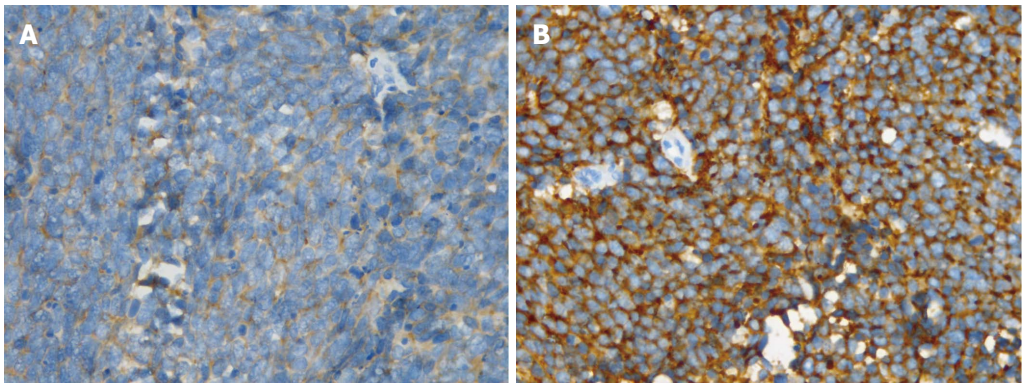

Figure 4 Immunohistochemical staining for synaptophysin. A: Immunohistochemical staining revealed positive expression of synaptophysin (SYN); B: Immunohistochemically, the tumor was positive for SYN.

decreased to the normal range one week after surgery and the levels of other tumor makers were maintained within normal ranges. The patient made an uneventful recovery and received combination chemotherapy with cisplatin and etoposide. The regimen consisted of cisplatin 25 mg/m^2 given intravenously on days 1-3 and etoposide 120 mg/m^2 intravenously on days 1-3, repeated every 4 wk. After 4 cycles of this regimen, treatment was discontinued due to serious neutropenia. Twelve months following surgery, a positron emission tomography-computed tomography (PET-CT) scan was normal and levels of tumor markers were well within normal ranges.

Case 2

A 34 year-old male presented to our hospital with a history of dull pain in the right upper quadrant of the abdomen for over 2 mo. There was no relationship between the pain and eating. The patient had no symptoms of dyspepsia or jaundice and the physical examination was normal. Full blood count, urea analysis, liver function tests and levels of tumor markers were within normal ranges. Two hyperecho masses in the gallbladder (27 mm × 14 mm, 31 mm × 16 mm) and dilation of the portal vein and common bile duct were revealed by abdominal ultrasonography. No stones were detected in the gallbladder. A CT scan of the abdomen revealed a polypoid intra-luminal lesion about 4 cm in diameter within the gallbladder and lymph node enlargement around the hepatic hilum and pancreas (Figure 1B). En bloc cholecystectomy and lymphadenectomy of the hepatic hilum and pancreas were performed. No distal metastasis or ascites was observed during the operation. Postoperative pathological findings showed MANEC of the gallbladder with serosal and lymph nodes invasion. No transitional areas were observed between the two different parts (Figure 2B). Immunohistochemically, the tumor was positive for CGA (Figure 3B), SYN (Figure 4B), CD56, HER2, CK and mucin (MUC) 1. Furthermore, the Ki-67 index was over 50%. The patient was discharged without complications, but he refused adjuvant chemotherapy. He returned 4 mo later, presenting with back pain. An abdominal CT scan showed lymph node enlargement in the retroperitoneum and hepatic hilum. Intraarterial chemotherapy was performed and the pain was relieved. The patient is now receiving the first cycle of chemotherapy consisting of cisplatin and etoposide.

DISCUSSION

NETs are classified into three broad histological categories based on tumor differentiation and grade according to the WHO Classification of Tumors of the Digestive

System in 2010: well-differentiated, low-grade (G1); well-differentiated, intermediate-grade (G2); and poorly differentiated, high-grade (G3). Tumor differentiation and grade often correlate with mitotic count and Ki-67 proliferation index. NETs are staged according to the AJCC tumor (T), node (N) and metastasis (M) staging system. Carcinoids of the stomach, duodenum/ampulla/jejunum/ileum, colon/rectum, and appendix have separate staging systems, but NETs of the gallbladder do not[7].

NEC is frequently combined with other histological carcinoma elements, such as adenocarcinoma or squamous cell carcinoma. MANEC is diagnosed only when both portions are more than 30% in the pathological examination[8].

The clinical presentations of most patients are nonspecific, and vague abdominal pain is the most common initial symptom[9]. Radiological tests including ultrasonography, CT scan, magnetic resonance imaging and PET-CT can reveal gallbladder masses indicative of gallbladder cancer. It is almost impossible, however, to preoperatively differentiate the NEC from other subtypes of gallbladder carcinomas. Pathological examination and immunohistochemical staining such as for CGA and SYN are required for the diagnosis of NEC.

As with other gallbladder carcinomas, NEC can readily invade the adjacent liver parenchyma and later cause biliary obstruction, making it challenging to detect at an early stage. Due to the aggressiveness of the tumor and lack of early symptoms, patients are often diagnosed at an advanced stage with lymph node invasion or liver invasion, and accordingly have a poor prognosis[5,10]. Both of the two patients in our report had no hormone-related symptoms, such as diarrhea, flushing or hyperglycaemia. Previous reports show that most NETs of the gallbladder had no carcinoid syndrome[11]. The proportion of carcinoid, which should be classified as well differentiated and lower grade neuroendocrine tumor, is much lower in the gallbladder than any other site in the gastrointestinal system. This may be another factor which contributes to the poor prognosis of NET of the gallbladder.

If the gallbladder specimen reveals only mucosal involvement on microscopic examination (Tis), or a pathologic report returns with identification of an incidental cancer with only submucosal or muscular invasion (T1), a simple cholecystectomy is adequate[12]. But for patients in advanced stages without distal metastasis, radical cholecystectomy and lymphadenectomy combined with hepatic resection should be performed to obtain adequate margins[5]. Improved outcomes have been realized with aggressive radical operative therapy in gallbladder carcinoma, and some reports have shown that patients with a locally invasive NEC of the gallbladder may benefit from aggressive surgical treatment, followed by adjuvant chemotherapy[13]. In patients with unresectable tumors, systemic chemotherapy is the treatment of choice[14].

Until now, there has been no standard indication for adjuvant therapy in patients with biliary neuroendocrine tumor and decisions on adjuvant therapy were made on the basis of clinical situations such as dissemination state, resection margin and histological classification[9].

For patients with poorly differentiated NEC, cisplatin or carboplatin and etoposide are generally recommended as the primary treatment, representing one of the standard regimens employed for the treatment of small cell lung cancer[15]. Iwasa *et al*[15] concluded that the use of the cisplatin and etoposide combination as a first-line chemotherapy for hepatobiliary or pancreatic poorly differentiated neuroendocrine carcinoma had only marginal antitumor activity and relatively severe toxicity compared with previous studies on extrapulmonary poorly differentiated neuroendocrine carcinoma treated with the same regimen. Evolving data suggest that patients with small cells or extremely high Ki-67 index have a better response. Iype collected 29 cases of poorly differentiated NEC and concluded that the large-cell subtype had a worse prognosis than the small-cell variety and chemotherapy was more effective for the small-cell subtype[16]. Other regimens have also proved to be effective for NEC of the gallbladder. Taniguchi *et al*[17] reported a case of adenoendocrine cell carcinoma of the gallbladder which exhibited a partial response to the combined use of docetaxel and cisplatin, following the emergence of gemcitabine resistance.

Shimono *et al*[18] reported one case of large cell neuroendocrine carcinoma of the gallbladder in a 64-year-old woman who survived for 69 mo after the initial diagnosis due to the use of multimodal treatment, consisting of intraarterial chemotherapy, three-dimensional radiation therapy, right trisegmentectomy, and γ-knife irradiation (for brain metastases). This result proves that radiation therapy can be a useful modality for neoadjuvant and adjuvant therapy in achieving local control.

Biological targeted therapies (like SST analogs) have proved effective in controlling symptoms in patients with gastroenteropancreatic neuroendocrine tumors (GEP-NETs)[19]. Unfortunately however, no similar cases for the gallbladder have been reported to date.

With regard to prognosis, tumor invasion of adjacent structures is an important negative predictor of outcome, compared to the presence of localized gallbladder wall tumors which show better prognosis[8,20]. Evidence of elevated Ki67 and high mitotic index is also likely to be predictive of poor outcome much as has been described in other NETs[5].

In conclusion, NEC of the gallbladder is a rare subtype of gallbladder tumor with aggressive behavior and poor prognosis. Radical surgery, including en bloc cholecystectomy and lymphadenectomy combined with hepatic resection plus adjuvant chemotherapy are recommended for patients in advanced stages without distal metastasis. Radiotherapy should be considered to control local recurrence or metastasis. The standardization of treatment including surgery, chemotherapy, radiotherapy and biological targeted therapy still requires further investigations.

COMMENTS

Case characteristics

The two patients presented with non-specific symptoms including nausea, vomiting and dull pain.

Clinical diagnosis

Neuroendocrine carcinoma of the gallbladder.

Differential diagnosis

Pathological examination and immunohistochemical staining for chromogranin A (CGA) and synaptophysin (SYN) are the major methods for differential diagnosis.

Laboratory diagnosis

Preoperative serum carcinoembryonic antigen level was elevated in one patient.

Imaging diagnosis

A computed tomography scan of the abdomen revealed a gallbladder with thickened wall or an intra-luminal mass.

Pathological diagnosis

Immunohistochemical staining revealed positive expression of CGA and SYN.

Treatment

Both patients underwent radical surgery and chemotherapy consisting of cisplatin and etoposide.

Experiences and lessons

Radical surgery including en bloc cholecystectomy and lymphadenectomy, combined with hepatic resection plus adjuvant chemotherapy is recommended for patients in advanced stages without distal metastasis.

Peer review

The study is a very interesting report on 2 rare cases of neuroendocrine carcinoma of the gallbladder. The authors recommend radical surgery and adjuvant chemotherapy based on their experiences and previously published data. Immunohistochemical staining (*e.g.*, for pancreatic polypeptide and somatostatin) should be given more attention.

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CASE REPORT

Intestinal obstruction caused by extramedullary hematopoiesis and ascites in primary myelofibrosis

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Abstract

Primary myelofibrosis (PMF) is a clonal hematopoietic stem cell disorder. It is characterized by bone marrow fibrosis, extramedullary hematopoiesis with hepatosplenomegaly and leukoerythroblastosis in the peripheral blood. The main clinical manifestations of PMF are anemia, bleeding, hepatosplenomegaly, fatigue, and fever. Here we report a rare case of PMF with anemia, small bowel obstruction and ascites due to extramedullary hematopoiesis and portal hypertension. The diagnosis was difficult to establish before surgery and the differential diagnosis is discussed.

Key words: Primary myelofibrosis; Intestinal obstruction; Ascites; Extramedullary hematopoiesis

Core tip: Bowel obstruction caused by extramedullary hematopoiesis and ascites due to portal hypertension are uncommon symptoms in primary myelofibrosis. Physicians should bear in mind that these rare manifestations can occur at the same time in a single patient.

Wei XQ, Zheng ZH, Jin Y, Tao J, Abassa KK, Wen ZF, Shao CK, Wei HB, Wu B. Intestinal obstruction caused by extramedullary hematopoiesis and ascites in primary myelofibrosis. *World J Gastroenterol* 2014; 20(33): 11921-11926 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i33/11921.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i33.11921

INTRODUCTION

Primary myelofibrosis (PMF) is a clonal hematopoietic stem cell disorder characterized by bone marrow fibrosis, extramedullary hematopoiesis with hepatosplenomegaly and leukoerythroblastosis in the peripheral blood[1]. The clinical manifestations of PMF include severe anemia which is caused by ineffective erythropoiesis, bleeding, marked hepatosplenomegaly due to extramedullary hematopoiesis, hyperuricemia and constitutional symptoms such as cachexia, fatigue, and fever. Ascites may occur in PMF due to portal hypertension[2,3]. As extramedullary hematopoiesis can occur anywhere, the clinical manifestations can be diverse. Extramedullary hematopoiesis mimicking acute appendicitis and intestinal obstruction, rectal stenosis, gastric outlet obstruction and bladder outlet obstruction due to extramedullary hematopoiesis have been reported[4-8]. However, intestinal obstruction and ascites

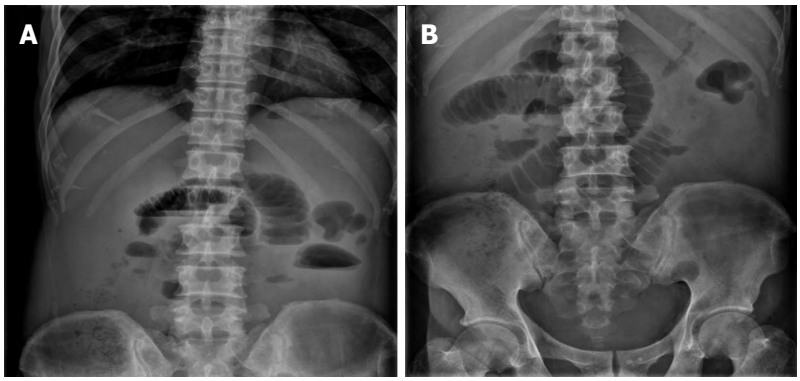

Figure 1 Plain abdominal radiography revealed incomplete small intestinal obstruction. A: The flat film of the abdomen in the standing position showed dilated small bowel loops with air-fluid levels; B: The flat film of the abdomen in the horizontal position showed dilated small bowel.

occurring at the same time in a patient with PMF has not been reported to date.

CASE REPORT

A 61-year-old man attended the Emergency Department of our hospital in October 2013 with complaints of significant weight loss, fatigue and anemia since May 2012. He underwent bone marrow biopsy on November 2012 and the results confirmed the diagnosis of PMF. In June 2013, the patient complained of vomiting, abdominal pain and abdominal distension with passage of flatus. He was then admitted to the Department of Hematology, where he underwent plain abdominal radiography which revealed incomplete small intestinal obstruction (Figure 1); computerized tomography (CT) scan and ultrasound B scan revealed hepatosplenomegaly and ascites, but no mass was found. Anal double-balloon enteroscopic examination was unremarkable, while peroral double-balloon enteroscopic examination was refused by the patient. After fasting for five days, the abdominal symptoms were relieved and the patient was discharged. The patient returned to our hospital with the chief complaints of vomiting, abdominal pain and abdominal distension, but with passage of flatus for a week. On physical examination, his vital signs were normal, however, pallor with ascites, hepatosplenomegaly and hyperactive bowel sounds with no palpable abdominal mass were observed.

Laboratory blood examinations showed the following indices (normal range in parentheses): hemoglobin, 59 g/L (120-140 g/L); peripheral white cell count, 5.39 × 10^9/L (5 × 10^9/L-10 × 10^9/L); neutrophils, 72.2% (40%-60%); peripheral red cell count, 2.47 × 10^{12}/L (4.0 × 10^{12}/L-4.5 × 10^{12}/L); platelet count, 210 × 10^9/L (100 × 10^9/L-300 × 10^9/L); peripheral eosinophil count, 0.02 × 10^9/L (0.02 × 10^9/L-0.52 × 10^9/L); C-reactive protein, 20.5 mg/L (0-6.0 mg/L); erythrocyte sedimentation rate, 31 mm/h (0-20 mm/h); albumin, 31.8 g/L (36-51 g/L); total immunoglobulin, 29.8 g/L (25-35 g/L); total bilirubin, 4.3 μmol/L (4-23.9 μmol/L); alkaline phosphatase, 57 U/L (35-125 U/L); c-glutamyl transpeptidase, 18 U/L (7-50 U/L); aspartate aminotransferase, 11 U/L (14-40 U/L); alanine aminotransferase, 5 U/L (5-35 U/L); creatinine, 192 μmol/L (31.8-91.0 μmol/L); blood urine nitrogen, 4.71 g/L (2.4-8.2 g/L); uric acid, 1108 μmol/L (90-420 μmol/L); prothrombin time, 14.3 s (11.0-14.5 s). Hepatitis B and C markers were negative. Serum tumor marker, cancer antigen 125 (CA125) was 321.2 U/mL (0-35 U/mL); alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA) and cancer antigen 19-9 (CA 19-9) were not elevated. Tuberculosis (TB)-related antibodies were not found in the blood, and the TB-purified protein derivative (PPD) skin test was negative. Antinuclear antibodies (ANA), antineutrophil cytoplasmic antibody (ANCA) and rheumatoid factor (RF) were not found in the blood. Routine urine and stool tests did not reveal any RBCs or proteins on the first day of hospitalization. Routine ascites test: color, yellow; Rivalta test, negative; red cell count, 280 × 10^6/L; red cell count, 66 × 10^6/L; lymphocytes, 70%; granulocytes, 30%; and no obvious eosinophilic granulocytes. The total protein, albumin, glucose, lactic dehydrogenase (LDH) and adenosine deaminase (ADA) levels in the abdominal fluid were 30.9 g/L, 18 g/L, 4.87 mmol/L, 95 U/L and 3.0 U/L, respectively. The serum-ascites albumin gradient (SAAG) = 31.8 g/L - 18 g/L = 13.8 g/L >11 g/L. No tumor cells were found in the ascites fluid following two cytopathology tests.

Abdominal CT scanning was repeated and revealed hepatosplenomegaly, huge ascites and thickened ileum wall with obvious enhancement in the arterial phase causing obstruction (Figure 2). However, it was difficult to determine whether the intestinal lesion was malignant or an inflammatory lesion, as there was an obvious enhancement in the arterial phase and ascites simultaneously.

During the first ten days of hospitalization, the patient received a nasogastric tube along with blood transfusion, albumin infusion, an intravenous proton pump inhibitor (pantoprazole 40 mg, twice daily), antibiotics and an intravenous diuretic (furosemide 20 mg, once or twice daily). Ascites reduced considerably, blood hemoglobin increased from 59 g/L to 89 g/L, blood creatinine decreased from 192 μmol/L to 116 μmol/L, and serum albumin increased from 31.8 g/L to 37 g/L, but there was no relief of abdominal symptoms.

The patient was then referred to the Department of Gastrointestinal Surgery, where a laparotomy and partial

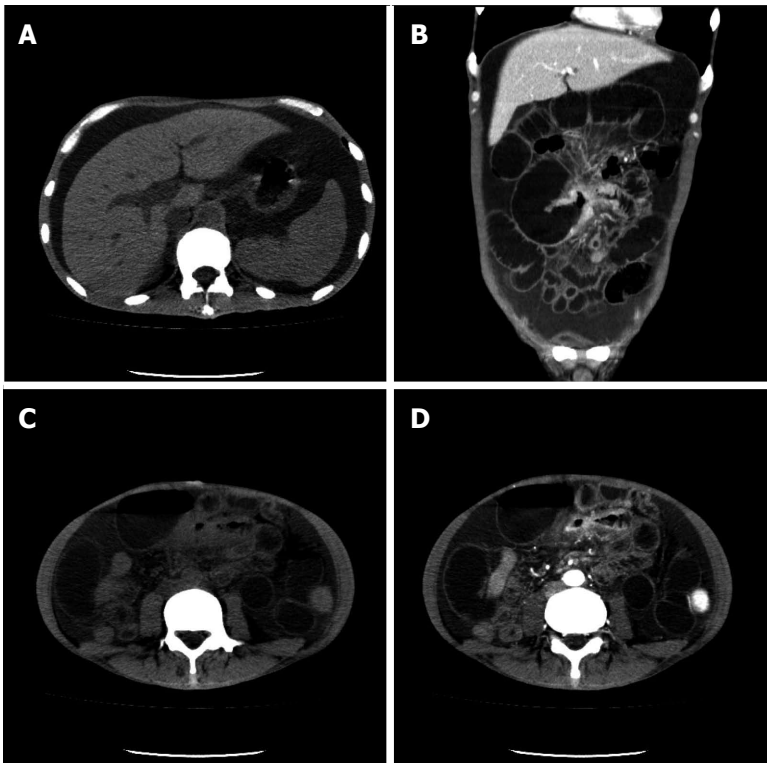

Figure 2 Computerized tomography images indicated hepatosplenomegaly and ascites (A) and the presence of thickened intestinal wall with obvious enhancement in the arterial phase and dilated small bowel (B-D). A: Plain computerized tomography (CT) scan; B: Coronal CT scan; C: Plain CT scan; D: The arterial phase.

enterectomy were performed. During surgery, an intestinal mass of approximately 5 cm × 3 cm × 3 cm and an obstruction in the ileum about 130 cm from the ileocecal valve were found, and intestinal adhesion forming a closed loop due to the mass was also observed. The resected ileum was 15 cm in length with a 5 cm × 3.5 cm yellow ulcerated mass in the center (Figure 3).

The pathological results were as follows: A gross view revealed a 5 cm × 3.5 cm brownish-yellow ulcerated mass in the 15 cm resected intestine. A microscopic view confirmed an ulcer in the resected specimen and there was significant hyperplasia of blood vessels in the deep layers of the intestine under the ulcer, along with hyperplasia of vascular endothelial cells. A large number of infiltrated inflammatory cells could be seen in the wall of the intestine; and a significant quantity of Megakaryocytes was observed around the serosal area along with an accumulation of immature myeloid cells and erythroid cells. Immunohistochemical (IHC) examination showed the following: CD61 (+), CD68 (-), MPO (+), CD34 (+), CD31 (+), CD11 (-), and Ki-67 (30%). An extramedullary hematopoietic mass of the small intestine with an ulcer and excessive vascular proliferation were confirmed pathologically (Figure 4).

Following surgery, the patient's abdominal symptoms and ascites completely resolved and he was discharged. Diuretics, testosterone undecanoate and thalidomide were prescribed in the outpatient department during a two-month follow-up and no abdominal symptoms were noted.

DISCUSSION

Extramedullary hematopoiesis occurs in conditions with an increased number of circulating myeloid progenitor cells, such as in PMF. The most common sites of extramedullary hematopoiesis are the spleen, liver, kidneys and the adrenal glands[9]. However, other organs are occasionally involved, such as the gastrointestinal tract[4-7], skin[10], joints[11,12], posterior mediastinum[13,14], the pericardium[15], and the brain[16-18]. A hematopoietic mass can cause symptoms resulting from stricture of hollow organs and compression of adjacent structures. In this patient, the hematopoietic mass was adherent to adjacent structures which also played an important role in causing symptoms. Intestinal obstruction, rectal stenosis, gastric outlet obstruction and bladder outlet obstruction due to extramedullary hematopoiesis have been reported[4-8], and progressive paraplegia may develop when extramedullary hematopoiesis occurs in the epidural space[17,18]. This patient suffered from a closed loop intestinal obstruction due to both intestinal stenosis and adhesion to the adjacent intestine caused by an extramedullary hematopoietic mass. As a closed loop was formed, it is reasonable that the ileac lesion could not be reached by a double-balloon enteroscopic examination. The lesion was identified on CT scan before surgery. In addition to gastrointestinal endoscopic examinations, CT scanning can also serve as an important tool in identifying gastrointestinal lesions. However, there was obvious enhancement in the arterial phase which was consistent with significant hyperplasia of blood vessels as confirmed by the pathological results in this patient, and it was difficult to determine whether the intestinal lesion was malignant on the CT scan.

Ascites are found in some PMF cases, the main cause of ascites is portal hypertension[2,3], peritoneal or other ectopic hematopoiesis can also be the main cause[19-22],

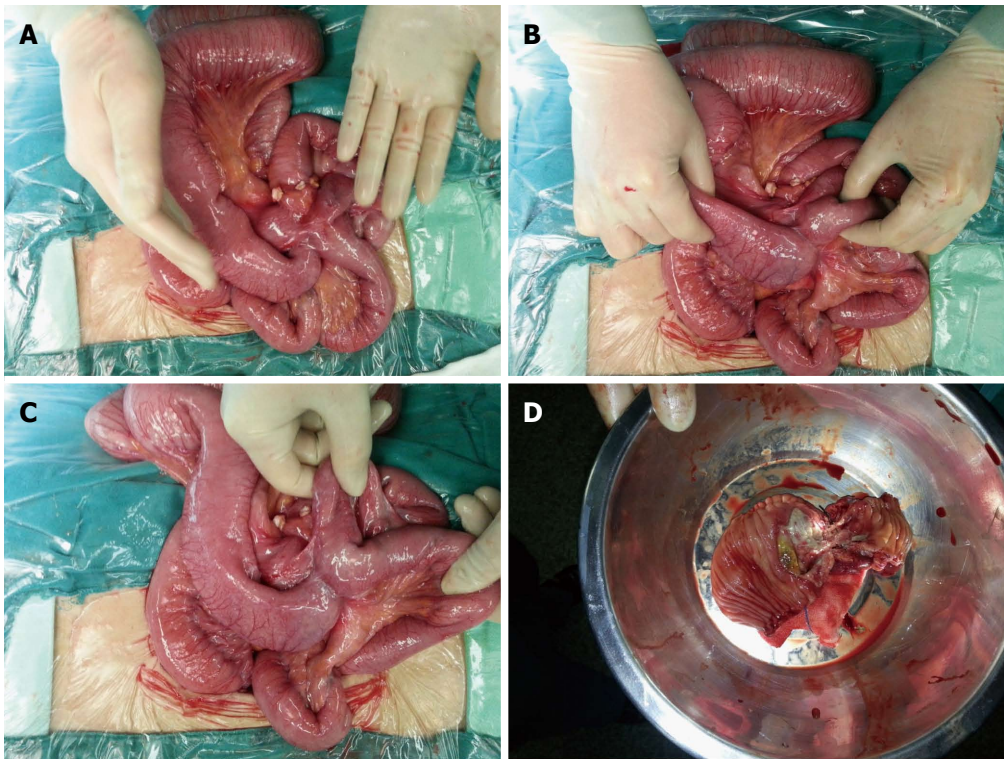

Figure 3 Exploratory laparotomy revealed intestinal obstruction caused by an intestinal mass approximately 5 cm × 3 cm × 3 cm and intestinal adhesions. A: The intestinal mass; B-C: Intestinal adhesions; D: The brownish-yellow mass with an ulcer.

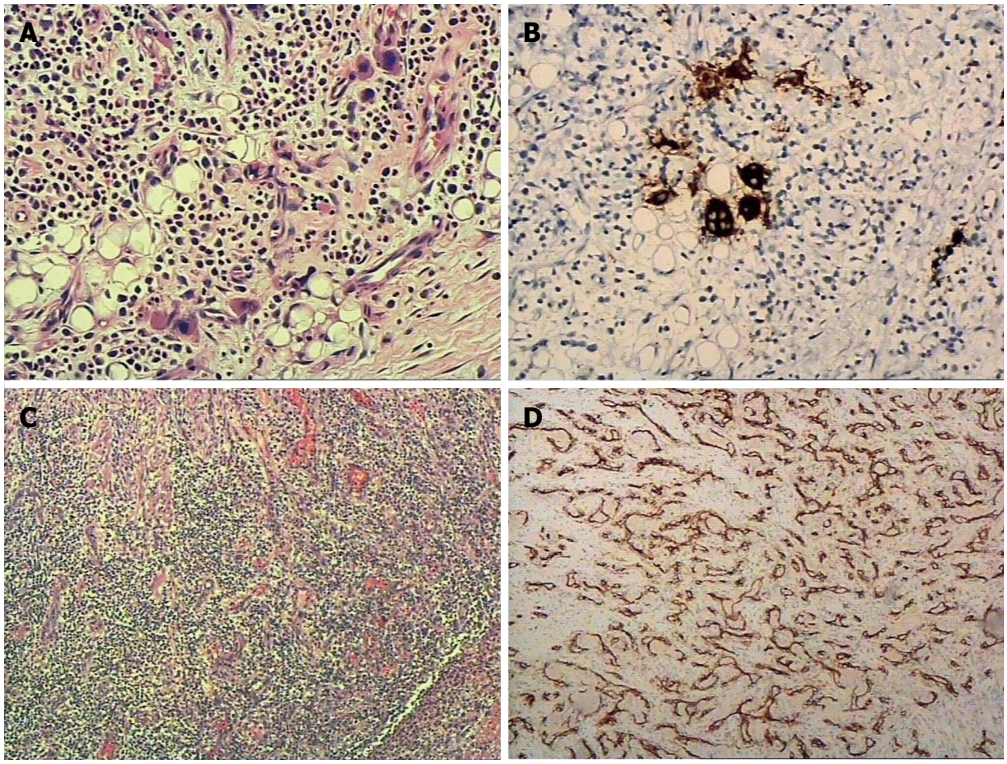

Figure 4 Extramedullary hematopoietic mass of the small intestine with ulcer and excessive vascular proliferation was confirmed by histopathology. A: A significant quantity of Megakaryocytes along with the accumulation of immature myeloid cells and erythroid cells were observed; B: Megakaryocytes were CD61 positive and CD68 negative (not shown); C: Significant hyperplasia of blood vessels and a large number of infiltrated inflammatory cells were seen; D: Blood vessels were confirmed by positive CD31 staining.

and hypoalbuminemia may play a role, as seen in this patient. Portal hypertension is due to two main mechanisms: firstly, increased blood flow through the massively enlarged spleen; secondly, functional intrahepatic obstruction caused by extramedullary hematopoiesis or periportal fibrosis in the liver[2,3,23,24]. Portal hypertension was proved indirectly by the high SAAG level which was higher than 11 g/L in this patient. The ascites, due to typical liquid leakage, reduced significantly following albumin infusion and intravenous diuretic before surgery, and were completely resolved by oral diuretics after surgery. Unfortunately, some patients with ascites are refractory to a sodium-restricted diet and high-dose diuretic treatment, TIPS may be a rescue therapy for refractory ascites secondary to portal hypertension, however, caution is necessary with respect to the presence and/or development of peritoneal or other ectopic hematopoiesis[3,19-22]. Ascites caused by peritoneal hematopoiesis have been reported to respond well to chemotherapy[19].

Intestinal obstruction and ascites occurring simultaneously in a patient is not rare, however, intestinal obstruction caused by extramedullary hematopoiesis and ascites occurring at the same time in a patient with PMF has, to our knowledge, not been reported. A series of differential diagnoses should be considered. Firstly, tuberculous peritonitis causing intestinal obstruction or intestinal tuberculosis causing intestinal obstruction with tuberculous peritonitis have been reported previously, and it is possible that abdominal tuberculosis occurs in PMF; however, there were no symptoms of tuberculosis, tuberculosis (TB)-related antibodies were not found in the blood, the TB-purified protein derivative (PPD) skin test was negative, and ascites was not an inflammatory exudate; thus, tuberculous peritonitis and intestinal tuberculosis were not diagnosed. Secondly, gastrointestinal carcinoma with intestinal stenosis causing metastatic ascites[25] or malignant ascites, such as peritoneal mesothelioma, causing intestinal obstruction[26-28] should also be considered. Fortunately, in this patient, blood CEA was normal, the ascites was due to typical liquid leakage, and no tumor cells were found in the ascites. These results revealed that the patient did not have a tumor. Thirdly, some autoimmune diseases such as systemic lupus erythematosus (SLE) can cause both intestinal obstruction and ascites[29,30]; however, in this patient, ANA, RF or ANCA were not found in the blood and the ascites was not an inflammatory exudate; thus, the diagnosis of autoimmune diseases was not considered. Fourthly, eosinophilic gastroenteritis presenting with intestinal obstruction and ascites has been reported[31,32]. Talley *et al*[33] identified three main diagnostic criteria for eosinophilic gastroenteritis: (1) the presence of gastrointestinal symptoms; (2) biopsies demonstrating eosinophilic infiltration of one or more areas of the gastrointestinal tract; and (3) no evidence of parasitic or extraintestinal disease; taking the normal level of eosinophilic granulocytes in the blood and ascites and a normal double-balloon enteroscopic examination into consideration, eosinophilic gastroenteritis was not a reasonable diagnosis. Lastly, bloody ascites caused by strangulation obstruction is a common clinical emergency, and was easily excluded in this patient.

Clinical physicians should bear in mind that intestinal obstruction caused by extramedullary hematopoiesis and ascites due to portal hypertension can occur at the same time in PMF, although it is not very common. Ascites due to portal hypertension may be resolved by diuretics, as in this case. Intestinal obstruction caused by an extramedullary hematopoietic mass can be cured surgically by removing the mass.

COMMENTS

Case characteristics

A 61-year-old man with a history of primary myelofibrosis presented with intestinal obstruction and ascites.

Clinical diagnosis

Intestinal obstruction caused by extramedullary hematopoiesis and ascites due to portal hypertension in primary myelofibrosis.

Differential diagnosis

Abdominal tuberculosis, abdominal cancer, autoimmune disease, and eosinophilic gastroenteritis.

Laboratory diagnosis

Hemoglobin 59 g/L; tuberculosis-related antibodies negative; CEA negative; and SAAG 13 g/L.

Imaging diagnosis

Computerized tomography scan revealed hepatosplenomegaly, ascites and a thickened ileum wall with obvious enhancement in the arterial phase causing obstruction.

Pathological diagnosis

Extramedullary hematopoietic mass of the small intestine with ulcer and excessive vascular proliferation was confirmed by HE staining and immunohistochemistry.

Treatment

A partial enterectomy was performed and diuretics were prescribed.

Term explanation

Extramedullary hematopoiesis is a phenomenon in which hematopoietic cells are found in sites other than the bone marrow.

Experiences and lessons

Laparotomy should be performed in patients with small intestinal obstruction of unknown cause in primary myelofibrosis.

Peer review

This paper presents a rare case of both intestinal obstruction caused by extramedullary hematopoiesis and ascites due to portal hypertension in primary myelofibrosis.

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Pregnant inflammatory bowel disease patients may require counselling regarding live vaccines in newborns

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Abstract

Inflammatory bowel disease patients are prone to immunosuppression due to effects of their medications. Physicians are recommended to assess vaccination status and overall health in all patients, prior to initiation of immunosuppressive therapy. Immunosuppressant medications in women with inflammatory bowel disease are often continued during pregnancy, which can result in newborns having an increased risk of immunosuppression at birth. While medication-induced immunosuppression in infants is transient, parents should be counselled about delaying live vaccine administration in newborns until they are immune competent. A retrospective study was done over six months at an urban multispecialty medical center to assess whether physicians are counselling pregnant immunosuppressed inflammatory bowel disease patients regarding live vaccinations in their newborns. The study revealed that only 57% of patients had documented counselling in their charts. Further studies are necessary to determine physician counselling practices of pregnant women about live vaccines. It is critical that physicians and patients are aware of the risks of immunosuppression in pregnancy and the potential impact of live vaccines upon the newborn.

Key words: Inflammatory bowel disease; Pregnancy; Vaccines

Core tip: International travel increases potential exposure to a variety of infectious agents. Administration of live vaccines may be important when traveling to certain areas. However, live vaccines pose risks for individuals who are immunosuppressed. This letter addresses the importance of counselling pregnant women with inflammatory bowel disease who are pharmacologically immunosuppressed about live vaccines and potential impact upon the newborn.

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TO THE EDITOR

We read with interest the case report authored by Sanchez-Tembleque *et al*[1] entitled "Vaccines and recommendations for their use in inflammatory bowel disease" which emphasized the importance of physicians analyzing vaccination status in all inflammatory bowel disease (IBD) patients prior to starting immunosuppressive therapy and the recommendation that live vaccines are contraindicated for the first six months in newborns with in utero immunosuppressant exposure. We are in agreement that physicians need to counsel pregnant mothers regarding the risks of live vaccinations in newborns. A report of a fatal outcome after BCG vaccination in a child born to a mother treated with Infliximab for refractory Crohn's em-

phasizes the importance of counseling about live vaccine administration[2,3]. Several studies have suggested some immune-modulating agents will cross the placenta and can alter the newborn's immune response[4,5]. While it is critical that infants receive appropriate immunizations, it is paramount that there is physician and patient recognition of the risks of live vaccination in immunosuppressed infants. Acquired neonatal immunosuppression from maternal IBD treatment may be under-recognized.

We conducted a six-month retrospective chart analysis evaluating the frequency of counseling about live vaccine risks in pregnant and post-partum women with inflammatory bowel disease maintained on immunosuppresants. There were no exclusion criteria. Seven patients (6 diagnosed with Crohn's disease and 1 with ulcerative colitis) either pregnant or breast feeding on immunosuppressive therapy were identified. The mean age was 32.3 years (range 22-36 years). There was 1 African American, 4 Caucasian, and 2 patients of unknown race. Five patients were on 6-MP, 1 infliximab, and 1 adalimumab. Four patients (57.14%) had documented discussions with their physician regarding potential effects of immunosuppressive therapy upon pregnancy. However, there were no documented discussions about the risks of live vaccines in a child born to a mother who is pharmacologically immunosuppressed. There was no statistically significant difference in the rate of such discussion based on diagnosis (P = 1.0), drug regimen (P = 0.58), or race (P =1.0).

The importance of immunization in children is well recognized. However, it is important that recipients of vaccines are informed of the risks associated with their administration. Potential risks of live vaccines are of particular concern in those who are immunosuppressed, including infants born to pharmacologically immunosuppressed mothers. Hence, it is imperative that physicians assess immune status, including the potential effect of medications upon immunity, prior to administering vaccines. While this study is limited based on size and duration, it suggests that physicians may not consistently discuss or may not consistently document their discussion about the risks of live vaccines. Increased efforts are necessary to ensure that physicians counsel women about vaccination risks in their newborns following intrauterine exposure to immunosuppressants.

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Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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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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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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